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Sequential changes in human Ito cells and their relation to postnecrotic liver fibrosis in massive and submassive hepatic necrosis

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Abstract To examine the relationship of Ito cells to postnecrotic liver fibrosis, liver specimens, obtained at autopsy from 17 patients with acute massive necrosis (AMN) and acute submassive hepatic necrosis (ASMN), were examined immunohistochemically. In normal adult livers, Ito cells positive for α-smooth muscle actin isoform (ASMA) were rarely seen, scattered along hepatic sinusoids. In contrast, in AMN the Ito cells in necrotic areas became strongly positive for ASMA. They were swollen with elongated cytoplasmic processes along collapsed sinusoidal walls. Around these ASMA-positive Ito cells, there were numerous infiltrated macrophages and lymphocytes present. There was no significant alteration of fibroblasts in the portal tracts. In the middle and late stages of ASMN, the spindle-shaped ASMA-positive Ito cells formed a continuous cellular network. New fibre formation was predominantly around them. In this immediate postnecrotic fibrosis, ASMA-positive stromal cells of Ito cell origin were distributed irregularly and were closely associated with reticulin and newly-formed collagen fibres. Regenerative nodules were surrounded by dense layers of ASMA-positive stromal cells. Throughout the stages of ASMN, portal fibroblasts remained negative for ASMA. We believe that Ito cells in necrotic areas show myofibroblastic transformation and play a central role in the postnecrotic liver fibrosis. Portal fibroblasts play no significant part in this type of fibrosis.

Key words Ito cell · Fulminant hepatitis · Postnecrotic fibrosis · α-Smooth muscle actin · Immunohistochemistry

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Introduction

Acute severe hepatitis, clinically diagnosed as fulminant hepatitis, is a life-threatening disease, usually associated with acute massive (AMN) and submassive hepatic necrosis (ASMN). The diseased livers show extensive and confluent parenchymal necrosis, collapsed sinusoidal structure and infiltration of numerous macrophages and lymphocytes in the early stages. In intermediate stages, ductal and ductular proliferation, cholestasis and postnecrotic diffuse fibrosis are seen. Finally, regenerative nodules develop, resulting in postnecrotic cirrhosis [2]. There have been no reports on sequential changes in Ito cells in relation to postnecrotic fibrosis in human livers, while a significant role of Ito cells has been confirmed in experimental liver fibrosis [13, 14, 16, 18, 20, 22, 23, 25]. This difference in the success of studies between man and experimental animals has arisen from difficulties in identifying Ito cells on routine histological sections of human livers at autopsy and in obtaining the livers of patients with fulminant hepatitis by needle and/or wedge biopsies. We have previously demonstrated that α-smooth muscle actin (ASMA) is a useful phenotypic marker of Ito cells in adult human livers. Furthermore, the Ito cells are more intensely stained with anti-ASMA antibody in association with liver cell necrosis and fibrosis [6, 7, 18, 21, 22, 27].

In this study using anti-ASMA antibody, we examined the Ito cells in liver specimens of autopsied patients with different stages of fulminant hepatitis which showed varying degrees of liver cell necrosis.

Materials and methods

Liver autopsy specimens of 17 patients with fulminant hepatitis were studied. There were 11 men and 6 women, 37 to 82-years-old (mean=56 years). The patients had clinical and biochemical features of acute hepatic necrosis with hepatic failure. Eleven patients had fulminant hepatitis in association with blood transfusion. Seven patients were seropositive for hepatitis B surface antigen. They died between the 7th and 191th day of illness. Autopsies were performed within 12 h of death.

As controls, 5 adult cases were selected from an autopsy series in our department. They had normal liver function tests and died of other diseases.

The liver weights varied from 450 g to 2000 g. Pathological diagnoses of the livers, based on the classification described by Craig [2], were AMN, 3 cases; ASMN, middle stage, 2 cases; and late stage, 12 cases. Autopsy livers for normal control weighed within normal range and showed no significant histological abnormalities.

All liver autopsy specimens were routinely fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned. The sections were stained with haematoxylin-and-eosin and additional stains such as silver impregnation, Azan-Mallory, periodic acid Schiff (PAS), Elastica van Gieson, orcein, Berlin blue and Victoria blue and mounted in Eukitt (Kindler, Freiburg, Germany).

For immunohistochemical study, the deparaffinized and rehydrated sections were processed as described previously [6]. Briefly, incubation with the primary antibody for 1 h at room temperature was followed by the second antibody, biotin-conjugated rabbit anti-mouse immunoglobulin G F(ab')$_2$ fragment (Dako, Glostrup, Denmark; diluted 1:200) and finally by the avidin-biotin complex horseradish peroxidase reagent (Vectastain kit, Vector Laboratories, Burlingame, Calif., USA). Reaction products were visualized by incubation in the fresh 3,3'-diaminobenzidine tetrachloride (Dojin, Kumamoto, Japan) solution containing 0.01% hydrogen peroxide. The monoclonal antibodies directed against ASMA (clone 1A4; Dako), human macrophage (clone KP1; Dako), human T cell (clone UCHL1; Dako) and proliferating cell nuclear antigen (clone PC10; PCNA, Novocastra Laboratories, Newcastle upon Tyne, UK) and Ki-67, nuclear antigen associated with cell proliferation (clone MIB-1; Immunotech) [8], diluted 1:50, 1:100, 1:100 and 1:100, respectively, were used. The monoclonal antibody against Ki-67 was a pre-diluted form ready for use. Immunostaining with the antibodies of PCNA and Ki-67 was carried also on microwave-processed paraffin sections [9].

Controls for immunostaining specificity, substituted the primary antibody with normal mouse serum, were always negative.

Results

In normal control livers without pathological abnormalities, vascular smooth muscle cells and pericytes were strongly positive for ASMA, serving as positive control. Within liver lobules a discontinuous layer of ASMA-positive cells was seen along the sinusoidal wall (Fig. 1). The sinusoidal ASMA-positive cells were Ito cells. They were distributed irregularly within liver lobules and occasionally contained a few small vacuoles (Fig. 2). Many other Ito cells showed very weak or undetectable immunostaining. Other sinusoidal lining cells such as sinusoidal endothelial cells and Kupffer's cells were invariably negative for ASMA. Neither lymphocytes nor infiltrated macrophages nor liver parenchymal cells were stained with anti-ASMA antibody. Around central veins, there were some layers of ASMA-positive cells.

In AMN, liver showed acute red atrophy. Almost all liver parenchymal cells were necrotic, with features of coagulation necrosis. Within liver trabecula severe haemorrhage occurred. Numerous macrophages as well as large and small lymphocytes infiltrated the necrotic areas. Ceroid-containing macrophages were PAS-positive/amylase-resistant and stained with anti-human macrophage antibody. Most of infiltrated lymphocytes were positive for UCHL1. Some of large lymphoid cells were stained with anti-PCNA antibody. Silver impregnation for reticulin fibres demonstrated diffuse collapse of sinusoids. However, the perisinusoidal reticulin framework appeared not to be severely affected. In contrast, immunostaining with anti-ASMA antibody demonstrated drastic changes in Ito cells in the hepatocellular necrotic areas (Fig. 3). ASMA expression of the Ito cells was markedly enhanced when compared with that in control livers. They showed diffuse swelling and elongation of cytoplasmic processes (40 μm and 15 μm in greatest length and width, respectively). The cell contour was very irregular. The ASMA-strongly positive Ito cells contained no small vacuoles, even though they had more abundant cytoplasm than those in control livers (Fig. 4). With stronger ASMA-staining in the subplasmalemmal portion than in the endoplasm, a circumferential dark line of reaction products was seen (Fig. 5). These cellular changes were not detected in routine light microscopic preparations, even by meticulous examination. The ASMA-positive Ito

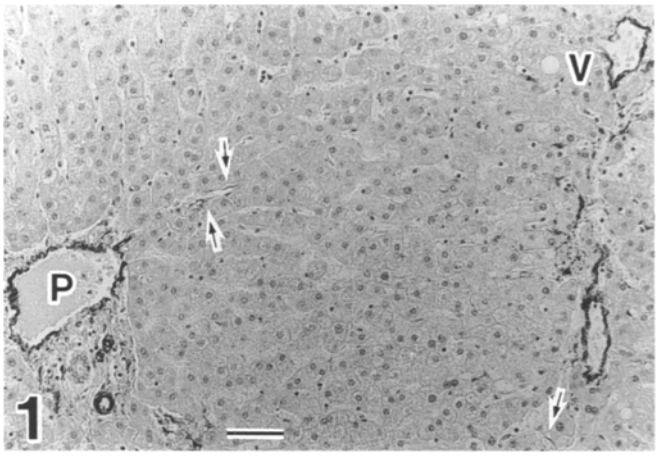


Fig. 1 α-Smooth muscle actin (ASMA) expression in normal control liver. In the portal tract (*P*) the walls of interlobular arteries and portal vein are positive for ASMA, while the fibroblasts are negative for it. Within liver lobule ASMA-positive Ito cells (*arrows*) are scattered along the sinusoid. V: central vein. Bar=50 μm, ×140

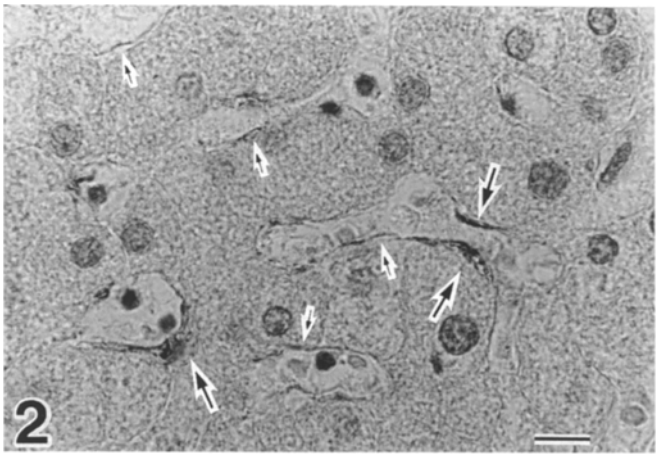


Fig. 2 Higher magnification of ASMA-positive Ito cells (*arrows*) in normal control liver. Note an occasional occurrence of their thin and linear perisinusoidal cytoplasmic processes positive for ASMA (*small arrows*). Bar=10 μm, ×680

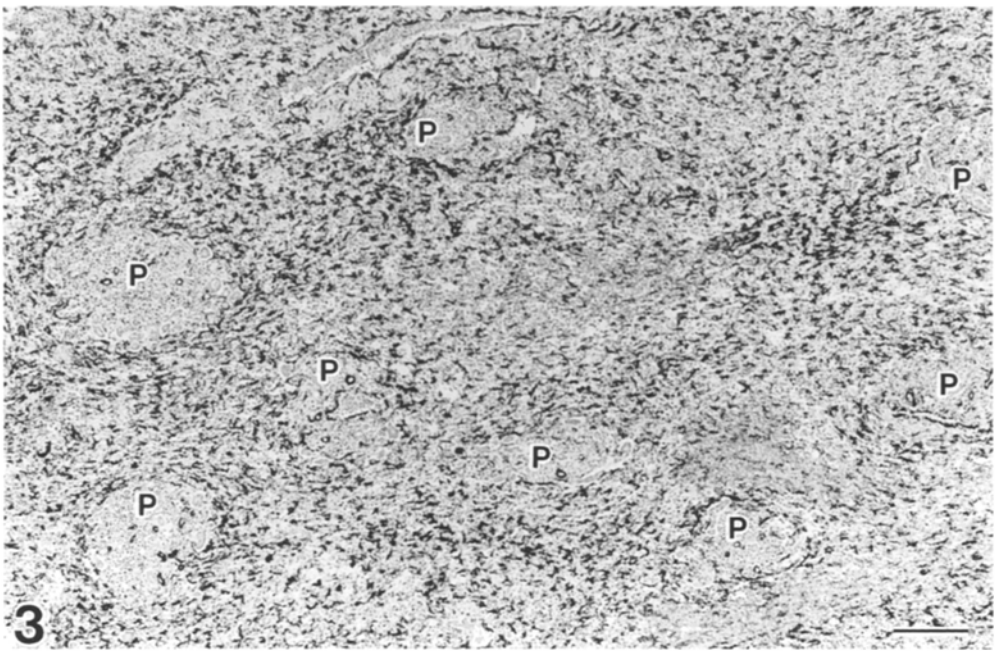


Fig. 3 Markedly enhanced ASMA expression of Ito cells in necrotic areas. Acute massive necrosis (AMN), at the 7th day of illness. Note diffuse swelling and increase in number of Ito cells. Adjacent portal tracts (*P*) with lymphocytic infiltrate appear close together. The fibroblasts remain negative for ASMA. Bar=200 μm, ×50

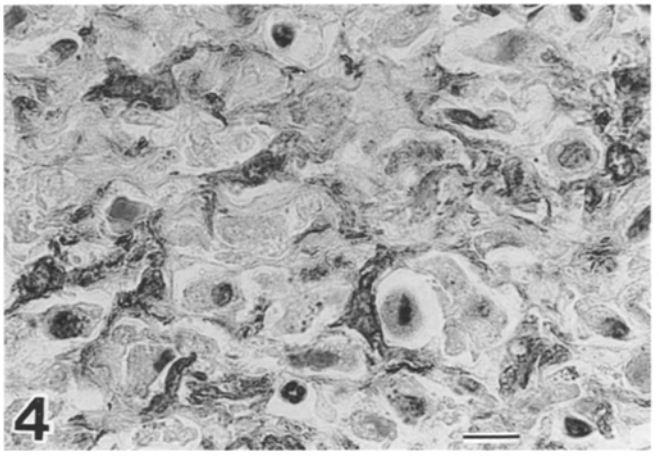


Fig. 4 Higher magnification of ASMA-positive Ito cells in necrotic area. AMN, at the 7th day of illness. They show irregular swelling and elongation of cytoplasmic processes as well as close association with infiltrated lymphocytes and macrophages. Bar=10μm, ×680

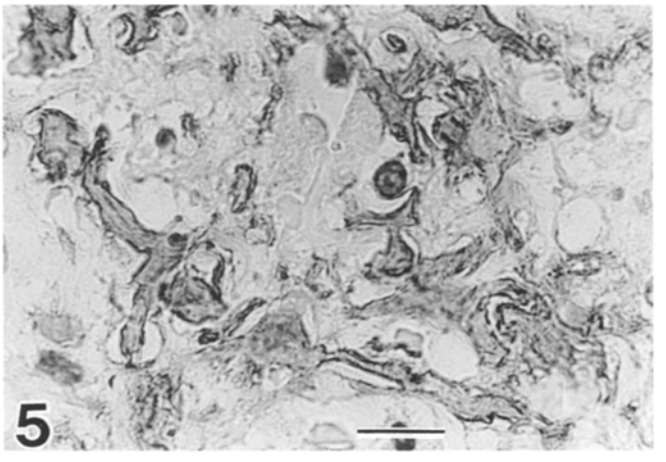


Fig. 5 ASMA-positive Ito cells in necrotic area. AMN, at the 11th day of illness. Note their elongated shape, irregular cell contour and linear margination by reaction products. Bar=10 μm, ×1080

cells showed no staining for both PCNA and Ki-67, while they had a close topographical relationship to infiltrated macrophages and lymphocytes.

In contrast to these intralobular changes, the portal tracts showed no significant alteration, except for mild oedema and lymphocytic infiltration. The fibroblasts in these areas were negative for ASMA.

In relatively early stage of ASMN, the increase in ASMA-expression was limited exclusively to the Ito cells in the necrotic area (Fig. 6). They were the same in shape and size as those in AMN. In contrast, Ito cells in non-necrotic areas resembled the staining pattern seen in normal livers.

With time after the onset of illness, necrotic debris was gradually removed and the inflammatory cells almost disappeared. At this stage the ASMA-positive Ito

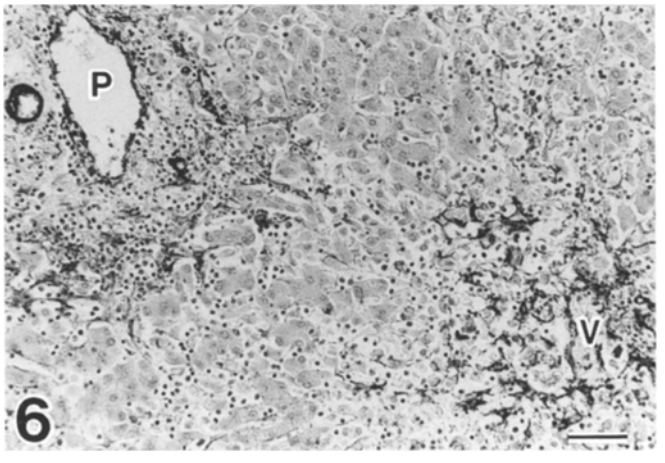


Fig. 6 ASMA-positive Ito cells in the perivenular (*V*) and partly periportal necrotic areas. Acute submassive necrosis (ASMN), at the 13th day of illness. *P*: portal tract. Bar=50 μm, ×140

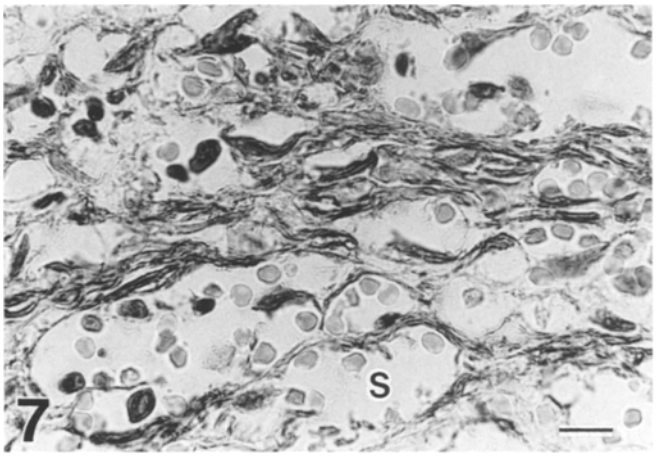


Fig. 7 ASMA-positive Ito cells in necrotic areas. ASMN, at the 27th day of illness. At this stage they are spindle-shaped and form a continuous cellular network along the sinsuoids (*S*). Necrotic debris is not yet seen. Bar=10 μm, ×680

cells became elongated and bipolar in shape. Their cellular network surrounded residual sinusoids circumferentially (Fig. 7).

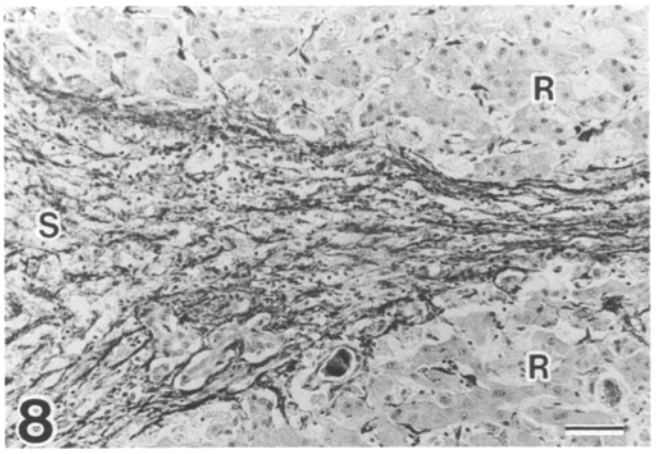
In the middle to late stage of ASMN, reticulin and newly-formed collagen fibres were increased around ASMA-positive Ito cells in the stroma, among the residual sinusoids. With progression of the fibrosis, the sinusoids became narrow-lumened and finally disappeared (Fig. 8). Small round regenerative nodules were seen in the loose fibrous stroma (Fig. 9), mostly sublobular in size. They were surrounded by fairly dense layers of ASMA-positive stromal cells (Fig. 10). Even where there was a long interval from the onset of the disease, the loose fibrous stroma was still present and invariably contained so many ASMA-positive stromal cells that it appeared to be uniformly stained intensely with anti-ASMA antibody at low magnification (Fig. 11). In such stroma, the ASMA-positive stromal cells were predominant and irregularly distributed, (Fig. 12) closely associated with reticulin and newly-formed collagen fibres. As shown in Fig. 13, the area of ASMA-positive stroma appeared to correspond well with new fibrotic areas. Rarely, small amounts of elastic fibres were also seen in the stroma. The portal tracts which maintained their basic structure were scattered in the broad fibrous stroma. At the interface between the portal tracts and loose fibrous stroma, varying degrees of proliferation of bile ducts and ductules were seen. ASMA-positive stromal cells were scattered among them. Ductular and ductal cholestasis were frequently seen. In contrast with the predominance of ASMA-positive stromal cells in surrounding stroma, there were few ASMA-positive Ito cells within regenerative nodules, if not associated with severe piecemeal necrosis.

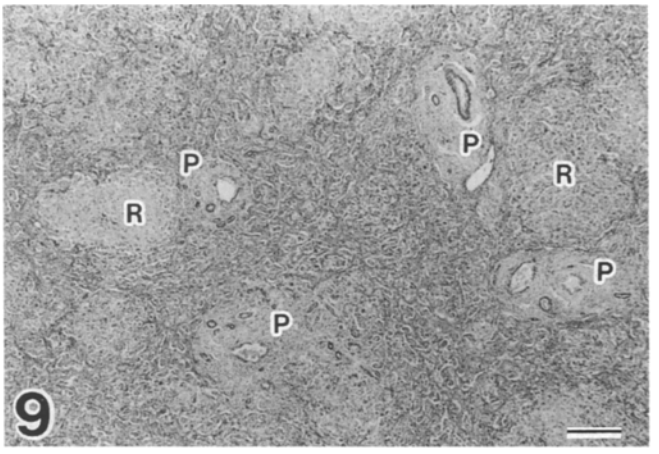
Throughout all stages of ASMN, the portal tracts showed no significant changes and were clearly distinguished from the loose fibrous stroma containing many ASMA-positive stromal cells. The portal fibroblasts remained negative for ASMA as did those in normal control livers.

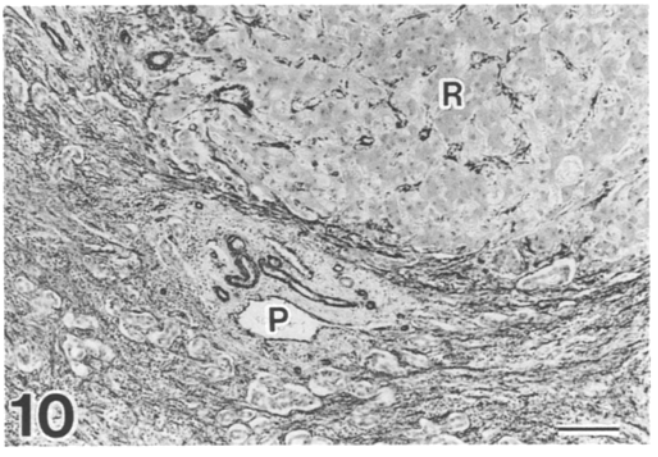
Discussion

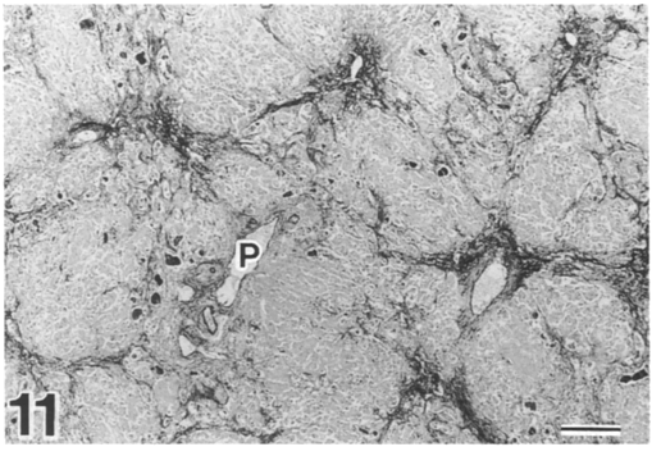
This study demonstrates drastic morphological and phenotypical changes of sinusoidal lining cells in hepatocellular necrotic areas in fulminant hepatitis. It was difficult, or rather impossible, to detect these changes on routine histological sections. A number of studies have demonstrated strong staining for ASMA along hepatic sinusoidal wall in various human liver diseases [6, 7, 18, 21, 27] and in experimental hepatic fibrosis [18, 20, 22, 23]. In these studies the sinusoidal lining cells positive for ASMA are considered to be activated or myofibroblastic Ito cells. By Northern blot analysis, ASMA gene expression increased with time in cultures of rat Ito cells [20]. Moreover, immunoelectron microscopic studies have clearly shown that among sinusoidal lining cells only Ito cells are positive for ASMA [6, 7, 22]. Therefore, staining for ASMA along hepatic sinusoidal walls is preferentially attributed to Ito cells.

In the early stage of the disease, the Ito cells locating in extensive necrosis showed a marked enhancement in ASMA-staining. From the staining pattern, their increase in number and changes including diffuse swelling, dendritic elongation of cytoplasmic processes with irregular cell contour and loss of intracytoplasmic vacuoles are easily recognized. All of the Ito cells in necrotic areas, regardless of its cause, may show hypertrophy. They exhibit myofibroblastic transformation, immunohistochemically identified by enhanced ASMA-expression [6, 22]. The stronger ASMA-staining of subplasmalemmal areas than that in perinuclear endoplasmic portion may reflect more numerous presence of ASMA-positive microfilaments and their longitudinal arrangement along cell axis in the ectoplasm [6, 22]. Although the ASMA-positive Ito cells are not stained with anti-PCNA and Ki-67 antibodies in this study, a possibility that post-mortem changes of autopsied livers may yield a false negative results, cannot be excluded [26]. In carbon tetrachloride-induced acute liver injury, swollen Ito cells in necrotic areas were strongly labelled by tritiated-thymidine [5]. Furthermore, in experimental liver fibrosis, the ASMA-positive Ito cells in the lobules and perifibrotic areas showed proliferative activities [23]. However, the Ito cells in the remaining liver parenchyma showed a similar ASMA-staining to that in normal livers. Thus, the activation of Ito cells is strictly confined to necrotic areas. Although the mechanism of Ito cell activation remains yet unclear, these findings strongly indicate that the most important aetiological factor is liver cell necrosis [6, 11, 21]. Through it, changes of extracellular matrix in Disse's space may influence the activity of Ito cells [15]. Following liver cell necrosis, infiltrated T lymphocytes and macrophages in necrotic areas may also stimulate adjacent Ito cells, possibly due to a paracrine mechanism [10, 19, 24]. In particular, transforming growth factor β1,









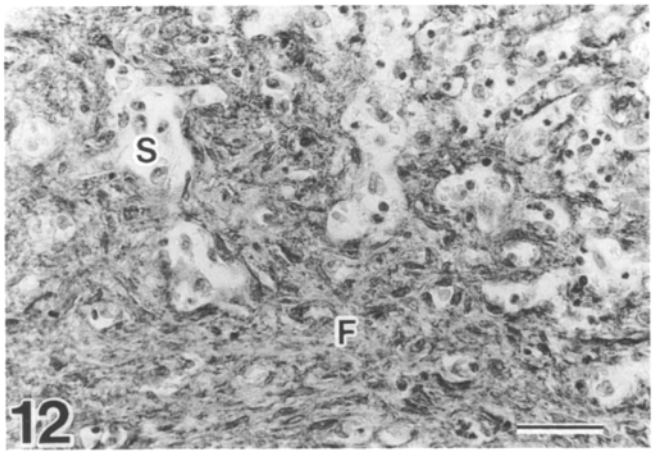


Fig. 8 Fresh fibrotic area between two regenerative nodules (*R*). ASMN, at the 34th day of illness. Among residual sinusoidal structure (*S*) in the left, the ASMA-positive Ito cells are irregularly distributed. Toward the right, with gradual disappearance of sinusoid they become elongated, condensed and run parallel with each other. Bar=50 μm, ×140

Fig. 9 Broad area of ASMA-positive loose stroma. ASMA, at the 34th day of illness. The portal tracts (*P*) are scattered in approximation with each other. *R*: regenerative nodule. Bar=200 μm, ×40

Fig. 10 A regenerative nodule (*R*) and a portal tract (*P*) are surrounded by loose fibrous stroma containing many ASMA-positive stromal cells. ASMN, at the 34th day of illness. The portal tract keeps its basic structure. The fibroblasts remain negative for ASMA. The periportal necrotic area is replaced by ASMA-positive fibrous tissue and associated with ductular proliferation. Bar=100 μm, ×70

Fig. 11 Late stage of ASMN, at the 55th day of illness. Areas of central-to-central and portal-to-central bridging fibrosis are strongly positive for ASMA. Ito cells in non-necrotic areas are negative for ASMA. *P*: portal tract. Bar=200 μm, ×40

Fig. 12 A marginal zone between areas of remaining sinusoids (*S*) and loose fibrous stroma (*F*). ASMN, at the 76th day of illness. The transition from ASMA-positive Ito cells to ASMA-positive stromal cells in the fibrous stroma is recognized. Bar=40 μm, ×270

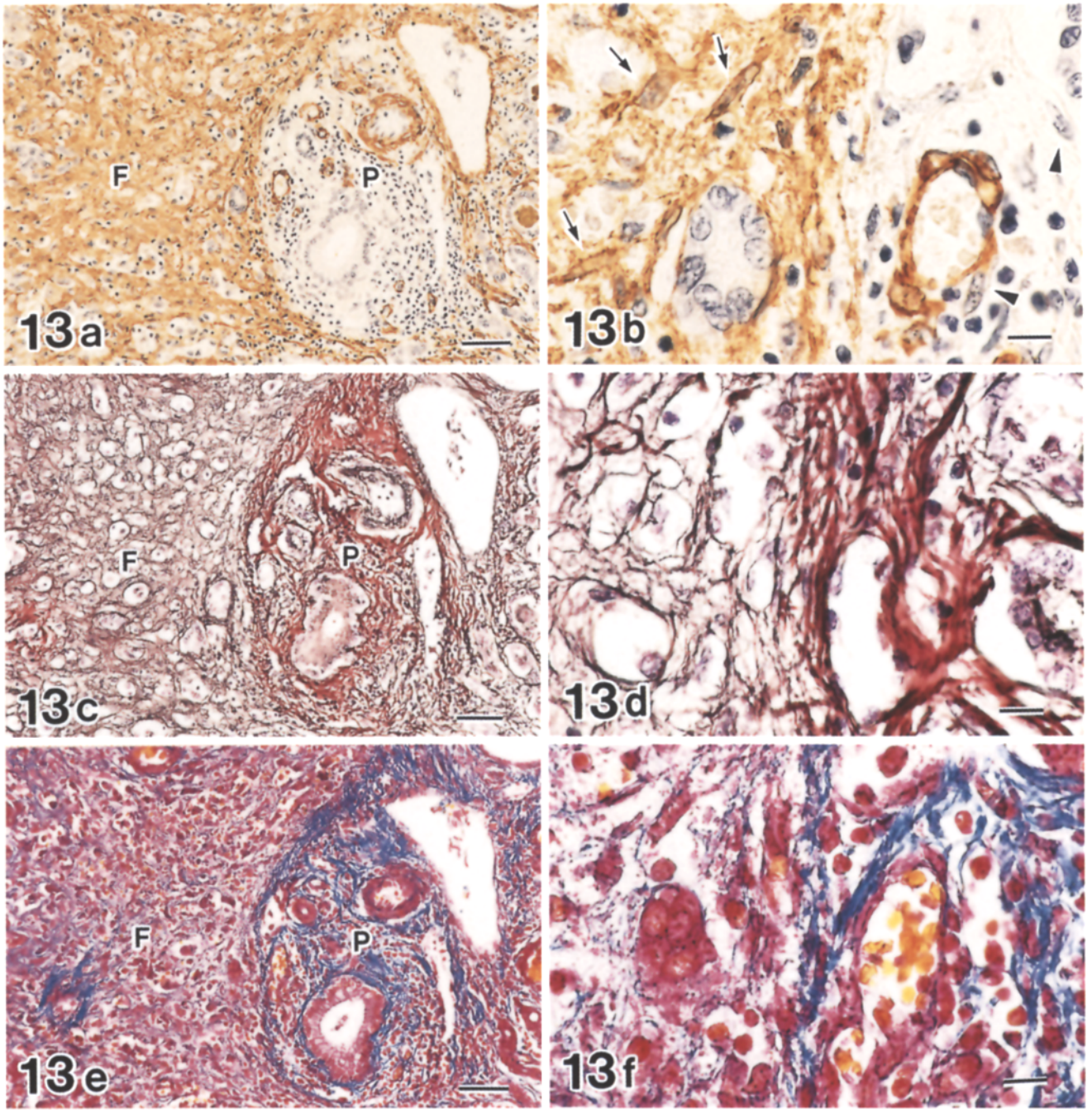


Fig. 13 A comparison of ASMA-staining (**a** and **b**), with silver impregnation (**c** and **d**) and Azan-Mallory stain (**e** and **f**) of serial sections. ASMN, at the 76th day of illness. Postnecrotic loose fibrous stroma (*F*) is predominantly composed of irregularly distributed and numerous reticulin fibres with immature collagen fibres together with a large number of ASMA-positive stromal cells (*arrows*). The portal tracts (*P*) include dense mature collagen fibres and a few reticulin fibres. The fibroblasts (*arrowheads*) are negative for ASMA. Fig. **b, d** and **f** exhibit the higher magnification of the interface between the portal tracts and the loose fibrous stroma. Fig. **a, c** and **e**. Bar=50 μm, ×140. Fig. **b, d** and **f**. Bar=10 μm, ×700

a potent cytokine secreted by infiltrated inflammatory cells and also by activated Ito cells themselves [17], is reported to induce ASMA expression in myofibroblasts and cultured fibroblasts [4] and to regulate extracellular matrix synthesis by human Ito cells [1].

With increasing time after liver cell necrosis, deposition of reticulin and collagen fibres was increased around the ASMA-positive Ito cells and a loose fibrous stroma was formed around them. The loose fibrous stroma gradually replaced the necrotic areas, resulting in the development of postnecrotic fibrosis. From observation of these sequential changes, the ASMA-positive stromal cells in loose fibrous stroma are considered to be derived

from activated Ito cells. Ito cells with the phenotypic features of myofibroblasts are clearly important in postnecrotic liver fibrosis, like those in chronic active hepatitis [12].

Dermal myofibroblasts which develop from local fibroblasts express ASMA transiently in the normal process of wound healing and actively produce extracellular matrix. But, they disappear from the scar tissue by the 30th day after wounding [3]. In contrast, ASMA-positive myofibroblastic stromal cells in postnecrotic liver fibrosis were seen even in the liver of the case autopsied at 191th day after the onset of illness. This may reflect continuing necrosis of liver cells and the progression of postnecrotic liver fibrosis.

However, the portal tracts showed no significant changes, except for inflammatory cell infiltrate and mild oedema. Clear distinction of portal tracts from areas of postnecrotic fibrosis was obtained not only by ASMA-staining, but also by routine connective tissue stains, as shown in Fig. 13. Furthermore, the fibroblasts in these areas remained negative for ASMA from early to late stage of the disease. These findings indicate that the portal fibroblasts have no major role in postnecrotic fibrosis.

In conclusion, in fulminant hepatitis the Ito cells preferentially locating in necrotic areas show sequential morphological and phenotypic changes after the onset of illness. In acute reactive phase, they undergo marked hypertrophy and hyperplasia which is recognized by their enhanced staining for ASMA and increase in number. These myofibroblastic Ito cells, in the following fibrotic phase, may produce reticulin and collagen fibres. Eventually, necrotic areas are replaced by newly synthesized fibrous tissue. In contrast, the portal fibroblasts have no or only a minor part to play in postnecrotic liver fibrosis.

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