MORPHOLOGY AND PATHOMORPHOLOGY

Ultrastructural and Immunohistochemical Study of Hepatic Stellate Cells over the Course of Infectious Viral Fibrosis and Cirrhosis of the Liver

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The dynamics of structural and functional changes in stellate cells in liver biopsy specimens (from lipid-containing to fibrogenous phenotype) was studied during the development of infectious viral fibrosis and cirrhosis of the liver using ultrastructural, immunohistochemical, and morphometric methods. The priority role of stellate cells in the synthesis of extracellular matrix components is emphasized. Resorption of perihepatocellular collagen fibrils is associated with parenchymatous liver cells.

Key Words: *fibrosis and cirrhosis of the liver; liver biopsy; hepatic stellate cells; ultrastructure; immunohistochemistry*

Fibrosis of the liver is a typical reaction to chronic liver injury caused by many factors, including ethanol, persistent viral infections, intoxication (including hepatotoxic drug treatment), hereditary disorders in metal metabolism [4,11,14].

A specific feature of fibrosis of the liver is the presence of dominants, special "growth points" of the connective tissue (portal zone, central zone of the lobule and Disse space) [13], determining the development of portal, central, and perihepatocellular fibrosis. The latter one is referred to most prognostically unfavorable variants, because it is associated with disorders in the trans-sinusoidal metabolism and metabolic function of hepatocyte.

Accumulation of extracellular matrix during development of liver fibrosis is not a static and/or unidirectional process, but a dynamic regulated process [7]. Progress in the study of liver cells clarified some cell bases of liver fibrosis: stellate cells of the liver (lipocytes or Ito cells) are now identified as the main source of extracellular matrix components in chronic liver diseases [5,9,15].

Stellate cells and other cells involved in fibrogenesis secrete a set of enzymes, matrix metalloproteinases, impairing the extracellular matrix. These enzymes destroy collagen and other matrix molecules and their presence in the connective tissue confirms the potentially dynamic nature of fibrotic processes in the liver. Molecular studies of mRNA expression for these enzymes (including enzymes with collagenolytic activity) showed that they are expressed in the liver even in cirrhosis, but their activity is confined to tissue inhibitors of metalloproteinases. Matrix degradation is possible even in pronounced cirrhosis, but it is impeded by competitive secretion of tissue inhibitors of metalloproteinases [8].

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We evaluated qualitative and quantitative characteristics of stellate cell population during the development of infectious viral fibrosis and cirrhosis of the liver.

MATERIALS AND METHODS

A complex pathomorphological study of 150 liver biopsy specimens from 104 patients with chronic HCV infection was carried out; 45 of these were specimens from patients with mixed HCV+HBV infection at different stages of fibrosis and cirrhosis of the liver. The stage of liver fibrosis was evaluated by a 4-point scale [6], from portal fibrosis to cirrhosis with the formation of porto-central vascularized septae and nodular transformation of the liver parenchyma.

Liver biopsy specimens were fixed in cold (4°C) 4% paraformaldehyde solution in Millonig phosphate buffer (pH 7.2-7.4). Paraffin sections were stained with hematoxylin and eosin in combination with Perls' reaction, after Van Gieson with poststaining of elastic fibers with Weigert resorcin-fuchsin, and PAS reaction was carried out. Semithin sections were stained with Schiff's reagent and Azur II. Ultrathin sections contrasted with uranyl acetate and lead citrate were examined under a JEM 1010 electronic microscope. The expression of smooth-muscle α-actin (NovoCastra Lab) in hepatic matrix-producing cells was tested by two-step indirect immunoperoxidase method with streptavidin-biotin system for visualization of the reaction product.

Numerical density of lipid-containing stellate cells per visual field (38,000 μ^2) was evaluated in semithin sections. The data were processed using Student's *t* test. The differences were considered significant at $p<0.05$.

RESULTS

Numerous stellate cells are usually observed in liver biopsy specimens from patients with chronic HCV infection; these cells are well seen only in semithin and ultrathin sections and can be differentiated in Disse spaces by the presence of lipid droplets in the cytoplasm. Transformation of stellate cells from passive (accumulating lipid-containing material)

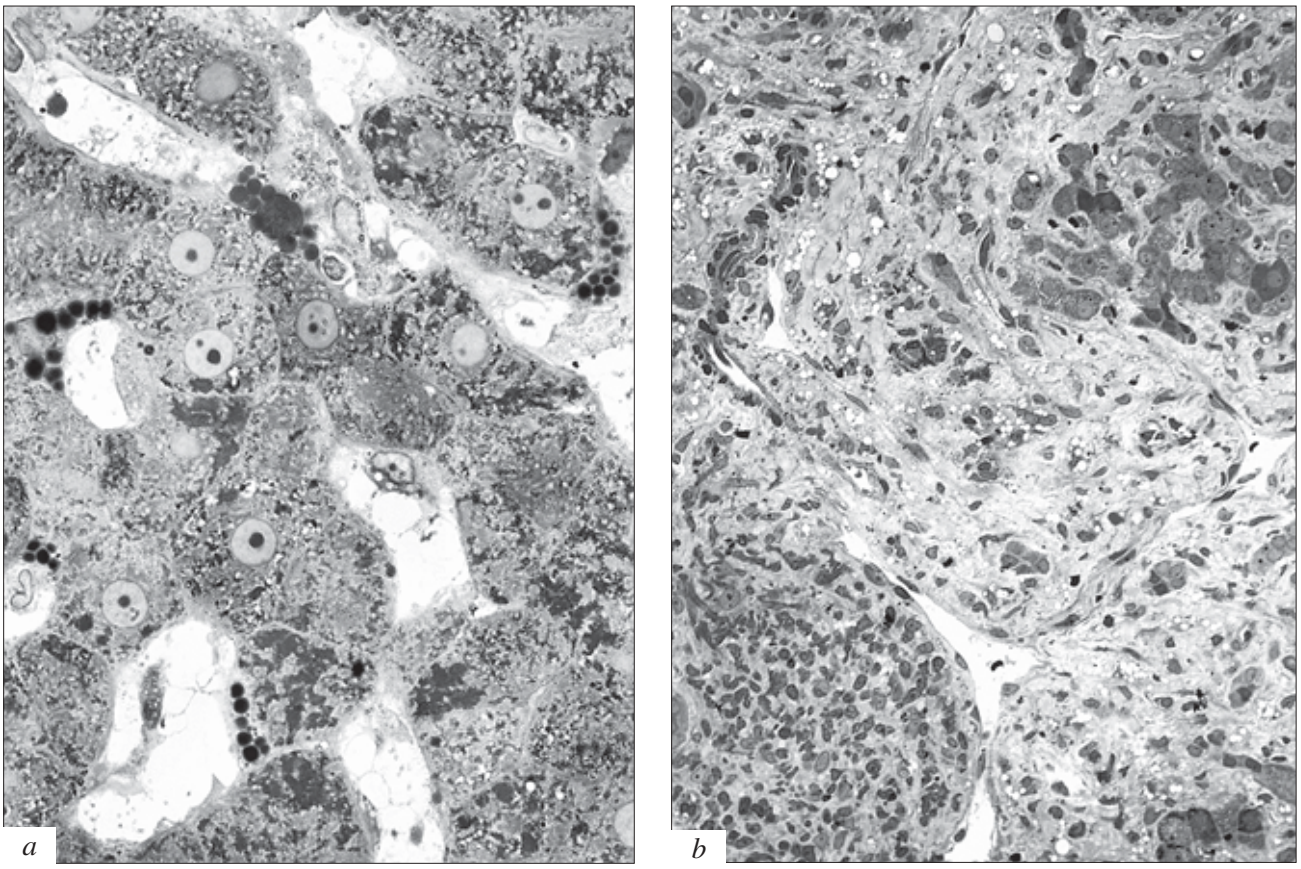

Fig. 1. Optic microscopic characteristics of liver stellate cells at different stages of infectious viral fibrosis. Semithin sections, staining with Schiff reagent and Azur II. *a*) numerous stellate cells in perisinusoidal Disse spaces. Lipid droplet polymorphism, minimum fibrosis stage (×1000); *b*) numerous lipid-containing stellate cells in periportal fibrosis zone. Liver cirrhosis stage (*300).

into fibrogenous is paralleled by disappearance of this main morphological marker. Therefore, the heterogeneous population of stellate cells can be quantitatively evaluated by complex ultrastructural and immunohistochemical analysis.

Liver stellate cells are characterized by pronounced polymorphism by the size, shape, number of lipid droplets, and their tinctorial characteristics (Fig. 1, *a*). At the initial stages of fibrosis (0, I), the numerical density of hepatic stellate cells discerned by the presence of cytoplasmic lipid droplets was 4.98±0.17 in chronic HCV infection and 2.45±0.06 cell in chronic HCV+HBV infection ($p<0.05$).

The numerical density of lipid-containing stellate cells decreased significantly at the stage of transformation into cirrhosis of the liver, this indicating fibrogenous transformation of these cells. However, sites with perisinusoidal lipid-containing stellate cells were seen in the liver parenchyma in solitary cases with cirrhosis. Moreover, numerous lipocytes were detected in the periportal fibrous tissue in one biopsy specimen (Fig. 1, *b*), which probably attests to an important role of stellate cells in retinoid metabolism in the body even at the stage of cirrhosis of the liver.

The data of ultrastructural analysis supplemented the microscopic characterization of stellate cells. Heterogeneous electron density of lipid granules, sometimes with the formation of electron more dense rim at the periphery, were noted (Fig. 2, *a*), as well as focal proliferation and emergence of binuclear lipocytes (Fig. 2, *b*).

With the progress of liver fibrosis, the ultrastructure of stellate cells acquired a so-called mixed or transitional phenotype with morphological signs of lipid-containing and fibroblast-like cell simultaneously. The nuclei in these lipocytes had deep invaginations of the nucleolemma, a larger nucleolus, and a greater cytoplasm, retaining lipid droplets. This was paralleled by an increase in the number of free ribosomes, polysomes, and granular cytoplasmic reticulum tubules. As a rule, membrane contact between lipid droplets and mitochondria was observed, indicating "utilization" of lipids (Fig. 2, *c*). Degradation of lipid droplets in some cells was realized by the formation of autophagosomes, which were later eliminated by exocytosis (Fig. 2, *d*).

Fibrogenous stellate cells are characterized by complete absence of lipid granules, elongated fibroblast-like shape, well-developed protein-producing compartment, and formation of contractile fibrillar structures in the cytoplasm. Numerous bundles of collagen fibrils formed extracellularly in Disse spaces in parallel with fibroblast-like transformation of stellate cells.

The degree of perihepatocellular fibrosis in chronic HCV infection was in significant negative correlation with the numerical density of lipid-containing stellate cells: their numerical density at fibrosis stage III and cirrhosis of the organ was 0.20±0.03 per visual field, which was significantly lower ($p<0.05$) than at fibrosis stages 0-I (4.98±0.17) and II (2.02±0.04).

Fibrogenous activity of matrix-producing cells was tested immunohistochemically by the expression of smooth-muscle α-actin. The products of immunohistochemical reaction of different intensity were detected in the cytoplasm of activated stellate cells, located inside the hepatic lobules. The most pronounced expression of smooth-muscle α-actin was observed in the cytoplasm of portal zone fibroblasts and myofibroblasts, vascular smooth-muscle cells, and myofibroblasts adjacent to the central veins.

On the whole, fibrogenous activation of stellate cells included a decrease in the number and subsequent disappearance of lipid droplets, focal proliferation of lipocytes, hyperplasia of the granular cytoplasmic reticulum, expression of fibroblast-like characteristics (including smooth-muscle α-actin), and formation of extracellular collagen fibrils (Fig. 3).

The study of paired biopsy specimens over the course of antiviral therapy showed the possibility of reducing fibrous changes in the liver (reduction of fibrous tissue volume in the perisinusoidal zones). By reconstruction of serial ultrathin sections we showed that resorption of collagen fibrils was realized by hepatocytes at the expense of expression of macrophagal characteristics [1,3].

Hence, the central event in the development of perisinusoidal fibrosis is activation of hepatic stellate cells, their transformation from passive cells accumulating vitamin A into contractile, proliferating, and fibrogenous cells. The major mediators of hepatic stellate cell activation are numerous cytokines and their receptors, LPO products, and other paracrine and autocrine signals [8].

Though the data on cellular mechanisms of fibrogenesis were primarily obtained in studies on hepatic stellate cells, it is obvious that different matrix-producing cells (each with special location, immunohistochemical and ultrastructural phenotype) make contributions of its own into the development of fibrosis of the liver [12]. These cells include portal tract fibroblasts and myofibroblasts, vascular smooth-muscle cells, and myofibroblasts adjacent to the central veins. Presumably, all matrix-producing cells are activated under conditions of chronic liver injury.

A hepatic stellate cell (intralobular mesenchymal cell) is characterized by unique origin, struc-

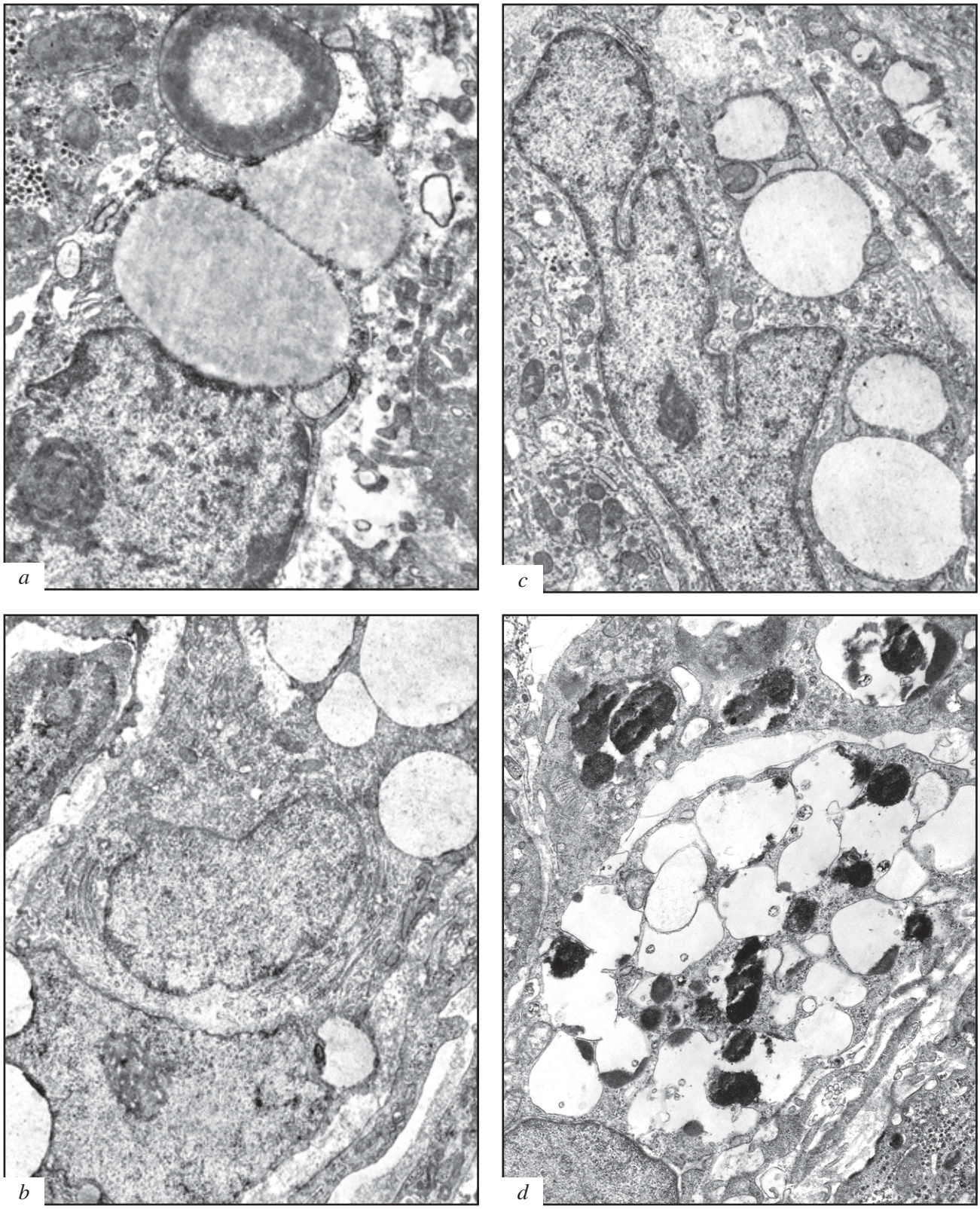

Fig. 2. Ultrastructural characteristics of liver stellate cells (electronograms). *a*) heterogeneous lipid droplets (×8000); *b*) binuclear stellate cell with numerous tubules of Golgi complex (×6000); *c*) deep invagination of the nucleolemma, mitochondrial contacts with lipid droplets, hyperplasia of protein-producing organelles (×8000); *d*) lipid droplet degradation (×6000).

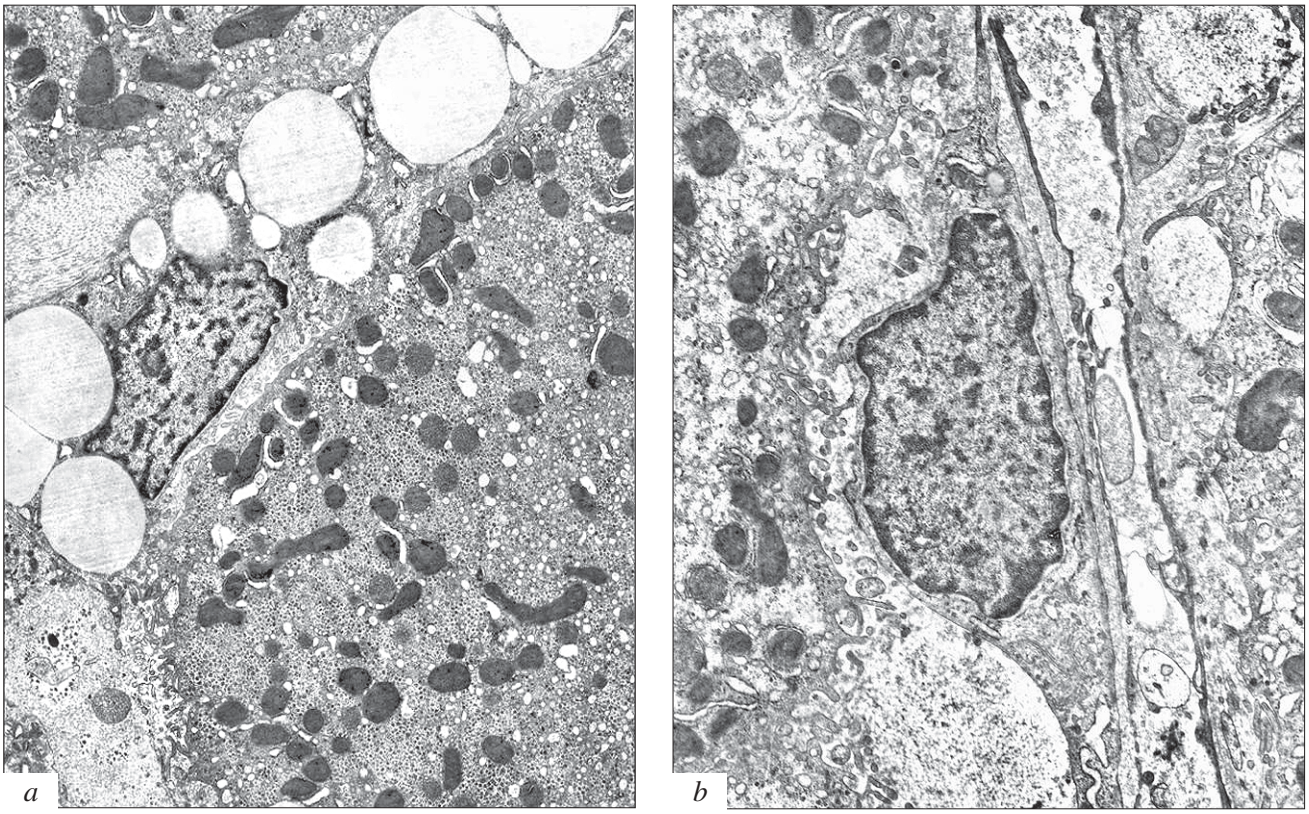

Fig. 3. Two contrast structural and functional phenotypes of liver stellate cells during development of liver cirrhosis (electronograms, ×4000). *a*) lipid-containing (silent) stellate cell in Disse space. Perisinusoidally: hepatocyte fragments; *b*) fibrogenous stellate cell: no lipid droplets; collagen fibrils in Disse space.

ture, and function [10]. One of the main functions of lipocytes is accumulation of vitamin A, these depots constituting more than 80% of total vitamin A content in the body, and the maintenance of vitamin A homeostasis [15]. Under conditions of disease, such as fibrosis and cirrhosis, hepatic stellate cells can be activated or "transdifferentiated" into the myofibroblast-like phenotype without cytoplasmic lipid droplets. Ito cell is the predominant intralobular liver cell producing components of extracellular matrix and metalloproteinases degrading them, which indicates their major role in remodeling of intralobular perihepatocellular matrix in health and disease. The structure and function of liver stellate cells are regulated by components of extracellular matrix, as well as by cytokines and growth factors *in vivo* and *in vitro*.

Stellate cells are also present in the pancreas, lungs, kidneys, and intestine [10]. Hepatic and extrahepatic stellate cells form disseminated system of stellate cells in the body, similar to the APUD system.

As hepatic stellate cells are the main cell type producing growth factors, cytokines, extracellular matrix components, and matrix metalloproteinases, they play leading role in the regeneration of the liver, including hepatocyte proliferation and remodeling of extracellular matrix.

The role of hepatocytes in reduction of perihepatocellular fibrosis is to be acknowledged in the context of phenomenon of extracellular matrix resorption in Disse spaces, detected in our study. We can hardly imagine the fibrogenesis process without participation of hepatic parenchymatous cells, as the participation of hepatocytes in resorption of the extracellular matrix collagen fibrils has been shown in experimental hepatocellular fibrosis [2] and in biopsy specimens from patients with viral hepatitis [1,3]. Hence, not only stellate cells and macrophages, but hepatocytes as well participate in the extracellular matrix remodeling.

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