Review paper

Liver sinusoidal endothelial cells in morphogenesis of pediatric autoimmune hepatitis. Ultrastructural characteristics – a novel report

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The pathogenesis of autoimmune hepatitis (AIH) is poorly understood. Up to now, little is known of the involvement of liver sinusoidal endothelial cells (LSECs), accounting for approximately 40% of nonparenchymal hepatic cells, in AIH morphogenesis in pediatric patients.

The study objective was ultrastructural analysis of LSECs from pretreatment biopsies of 19 children, aged 4-17 years (14 girls), with clinically and histologically diagnosed AIH.

Our study is the first to describe alterations in LSECs, from swelling to necrosis, demonstrating their important role in the morphogenesis and progression of pediatric AIH. Frequently damage to LSECs coexisted with significantly activated Kupffer cells, fibrogenesis and fibrosis, but not cirrhosis, accompanied by the appearance of transitional hepatic stellate cells. Interestingly, even though in half of the AIH children the sinusoidal vessels were found to undergo transformation of discontinuous into continuous endothelium showing features of defenestration, the true basement membrane did not form underneath. The fact that the basement membrane is not formed, even when LSECs are markedly damaged, may seem to indicate some regenerative capacities of these cells and lesion reversibility.

Key words: liver sinusoidal endothelial cells, nonparenchymal hepatic cells, pediatric autoimmune hepatitis, pretreatment liver oligobiopsy material, ultrastructure.

Introduction

Clinical manifestations of autoimmune hepatitis (AIH) – a chronic immune-mediated, autodestructive liver disease, requiring long-term immunosuppressive therapy, range from mild chronic to acute, sometimes fulminant hepatitis. Unfortunately, the pathological mechanisms of the disease are not yet fully understood because of the lack of suitable animal models [1, 2, 3, 4, 5, 6, 7].

It is assumed that liver biopsy is the gold standard in evaluating inflammation and fibrosis in AIH [1, 2, 3, 6, 8].

According to many authors, in AIH, the interface hepatitis is closely related to the process of liver fibrosis [4, 5, 6, 8, 9, 10, 11]. It has been emphasized that this autodestructive liver disease can result in cirrhosis, liver failure and death [2, 5, 10, 11, 12, 13, 14].

Recently, in the disease morphogenesis an increasing role has been ascribed to nonparenchymal hepatic

cells (NPCs), particularly Kupffer cells/macrophages (KCs/MPs) and liver sinusoidal endothelial cells (LSECs). Unfortunately, the research has been limited mainly to adult patients [9, 15, 16]. Apart from very few studies, including ours [17, 18], there are no similar reports referring to pediatric patients. It is especially important since although AIH in childhood is rare, it leads to cirrhosis more often than in adults [10, 11, 19, 20].

In our opinion, the involvement of LSECs, accounting for approximately 40% of the NPC population [21], in the pathogenesis and progression of AIH is extremely interesting, yet still not fully known. The more so as in our earlier ultrastructural studies on KCs/MPs in pediatric AIH we observed the coexistence of characteristic lesions within the population of Kupffer cells (glassy droplet inclusions within the cytoplasm of these cells) with marked damage to the endothelial lining of hepatic sinusoids [18]. This inspired us to perform more profound microscopic observations with LSECs.

It can be assumed that LSECs, also called liver sinusoidal endothelium (LSE) or endothelial lining, constituting the sinusoidal wall, are a highly specialized resident endothelial cell type with characteristic morphological and functional features. They represent the interface between blood cells on one side and hepatocytes and hepatic stellate cells (HSCs) on the other side [22, 23].

The liver sinusoids can be regarded as unique capillaries which differ structurally and functionally from other capillaries in the body, because of the presence of open pores or fenestrae clustered in sieve platelets lacking a diaphragm and a basal lamina underneath the endothelium [22, 24, 25]. Other ultrastructural characteristics of LSEC include the presence of numerous bristle-coated micropinocytic vesicles and many lysosome-like vacuoles in the perikaryon, indicating a well-developed endocytic activity [23, 24, 25, 27]. Discontinuous normal human LSECs differ also phenotypically from vascular or continuous endothelial cells, for instance in their failure to express platelet-endothelial cell adhesion molecule 1 (PECAM-1 or CD31), CD34, factor VIII-related antigen (FVIIIRAg), and E-selectin [23, 26, 27]. They have no basement membrane but only an attenuated extracellular matrix (ECM), consisting mostly of fibronectin [23, 24, 25, 27]. However, in the course of chronic hepatitis and cirrhosis, LSECs often undergo transformation into a vascular type-endothelial cells (capillarization of LSECs) showing features of defenestration with the formation of a true basement membrane, which may result in the development of hepatocellular failure and may have important clinical consequences [9, 22, 23, 27, 28, 29, 30].

Considering the above, especially the lack of similar morphological reports in pediatric patients, the current study objective was the ultrastructural analysis of LSECs in pretreatment liver biopsies obtained from children with clinicopatologically diagnosed AIH.

The study is a continuation of our electron-microscopic investigations on certain chronic liver diseases, including AIH, in pediatric patients [17, 18, 31, 32, 33, 34, 35]. It also refers to the observations of liver damage in various experimental models **[36,** 37**].**

Material and methods

Review of clinical and histopathological material

Ultrastructural analyses were performed on pretreatment biopsy liver specimens obtained from 19 children (5 boys and 14 girls), aged 4-17, hospitalized in the Department of Pediatrics, Gastroenterology, Hepatology, Nutrition and Allergology, Medical University of Bialystok, with clinically and histologically diagnosed AIH. Laboratory tests revealed markedly increased serum levels of aspartate and alanine aminotransferase in all study patients. Immunological and serological disturbances in the blood serum were manifested by elevated IgG levels, presence of autoantibodies – antinuclear antibodies (ANA) and/or smooth muscle antibodies (SMA). Differential diagnostics excluded, among others, infectious liver diseases (HBV, HCV, CMV, Toxoplasma gondii), some metabolic disorders (Wilson's disease, cystic fibrosis, 1-antitrypsin deficiency) and celiac disease.

All the children underwent percutaneous needle liver biopsies. The collected material was subjected to morphological, both histopathological and ultrastructural analyses using transmission electron microscope (TEM) in the Department of Medical Pathomorphology, Medical University of Bialystok.

The study revealed typical histological features of AIH, i.e. interface and lobular hepatitis, of moderate/severe degree, with mainly portal infiltration of lymphocytes and plasma cells, severe necroinflammatory reaction, and rosette formation of hepatocytes; the alterations were frequently accompanied by portal, periportal and bridging fibrosis [18].

Informed consent was obtained from parents of each patient included in the study. The current research was approved by the Ethical Committee, Medical University of Bialystok (R-I-002/410/2016).

Ultrastructural analysis

For ultrastructural investigations, small fresh liver blocks (1 mm^3 volume) were fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4, at room temperature. Subsequently, the specimens were

postfixed in 2% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, pH 7.4, for 1 h. Then, the material was dehydrated through a graded series of ethanols and propylene oxide, embedded in Epon 812 and sectioned on Reichert ultramicrotome (Reichert Ultracut S) to obtain semithin sections. Next, the sections were stained with 1% methylene blue in 1% sodium borate and routinely processed for TEM analysis and examined using an Opton 900 EM (Zeiss, Oberkochen, Germany) and photographed with TRS camera (CCD-Camera for TEM 2K inside). This processing procedure had been used in our earlier TEM investigations of the liver in pediatric patients [18, 32, 33, 34, 35]. LSECs were determined by a microscopist who was blinded to the clinical information.

Results

In all study children the ultrastructural analysis of the liver sinusoidal vessels in oligobiopsy material showed substantial morphological abnormalities of endothelial lining characterized by variously pronounced degenerative lesions, including necrosis (Figs. 1-4).

We observed substantial swelling of liver sinusoidal endothelial cells (Figs. 1A, B; 2B and 3A-C) and their protrusion to the vascular lumen, leading to its marked decrease (Figs. 1A, B and 2B). Swollen endothelial cells relatively frequently showed features of defenestration, i.e. contained a smaller number of oval fenestrae characteristic of normal liver sinusoidal endothelial cells, and in approximately half of the cases underwent transformation to continuous endothelial cells (i.e. showed a tendency towards transformation into vascular-type endothelial cells) (Fig. 3A-C). Interestingly, however, in the bioptates examined, the transformed continuous liver sinusoidal endothelium did not exhibit the formation of a true, organized basement membrane (Fig. 3A-C), i.e. features of completed vascularization. Sometimes within the continuous liver sinusoidal endothelial cells the formation of tight junctions was observed. Swollen endothelial cells contained enlarged nuclei (Fig. 1A, B), and a reduced number of intraplasmatic organelles, especially micropinocytic vesicles, undergoing dispersion and degeneration. Fragments of canals of granular endoplasmic reticulum, polysomes and free ribosomes, altered mitochondria and few phagolisosomes were identified in fine granular background. Residual cytoplasmic structures, mainly micropinocytic vesicles, were quite frequently located on the cell periphery. The cytoplasm of more swollen LSECs was electron-translucent and almost empty in places and quite frequently contained cistern-like vacuolar structures of various size (Figs. 2B and 3A-C). Sometimes swollen endothelial cells showed features of marked phagocytosis, which was reflected

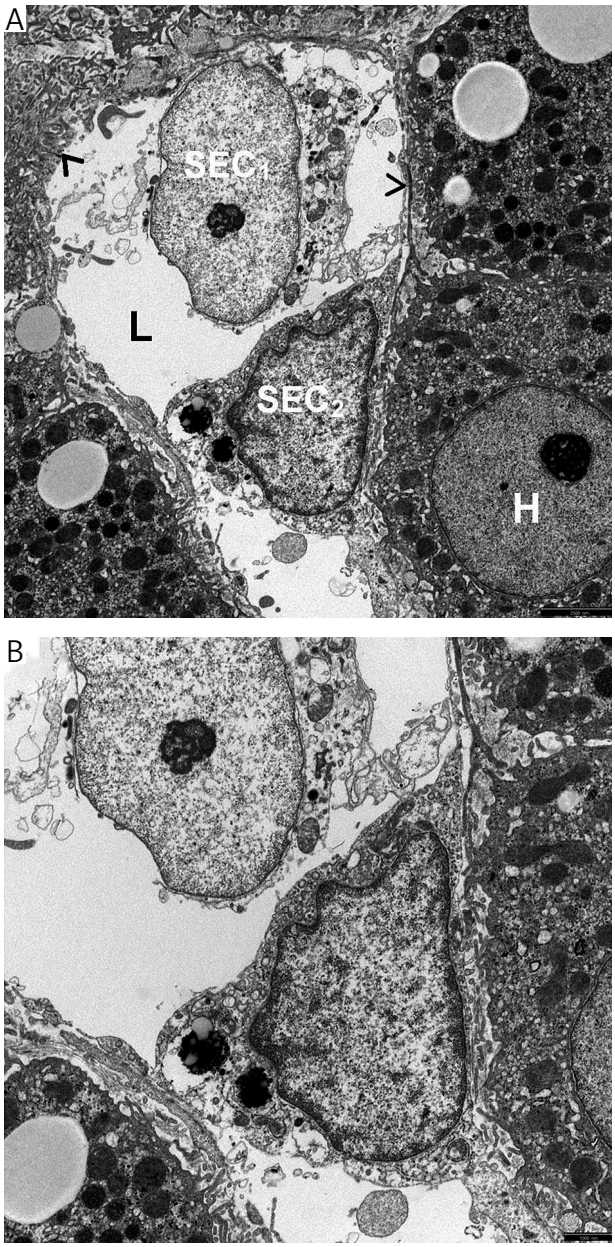
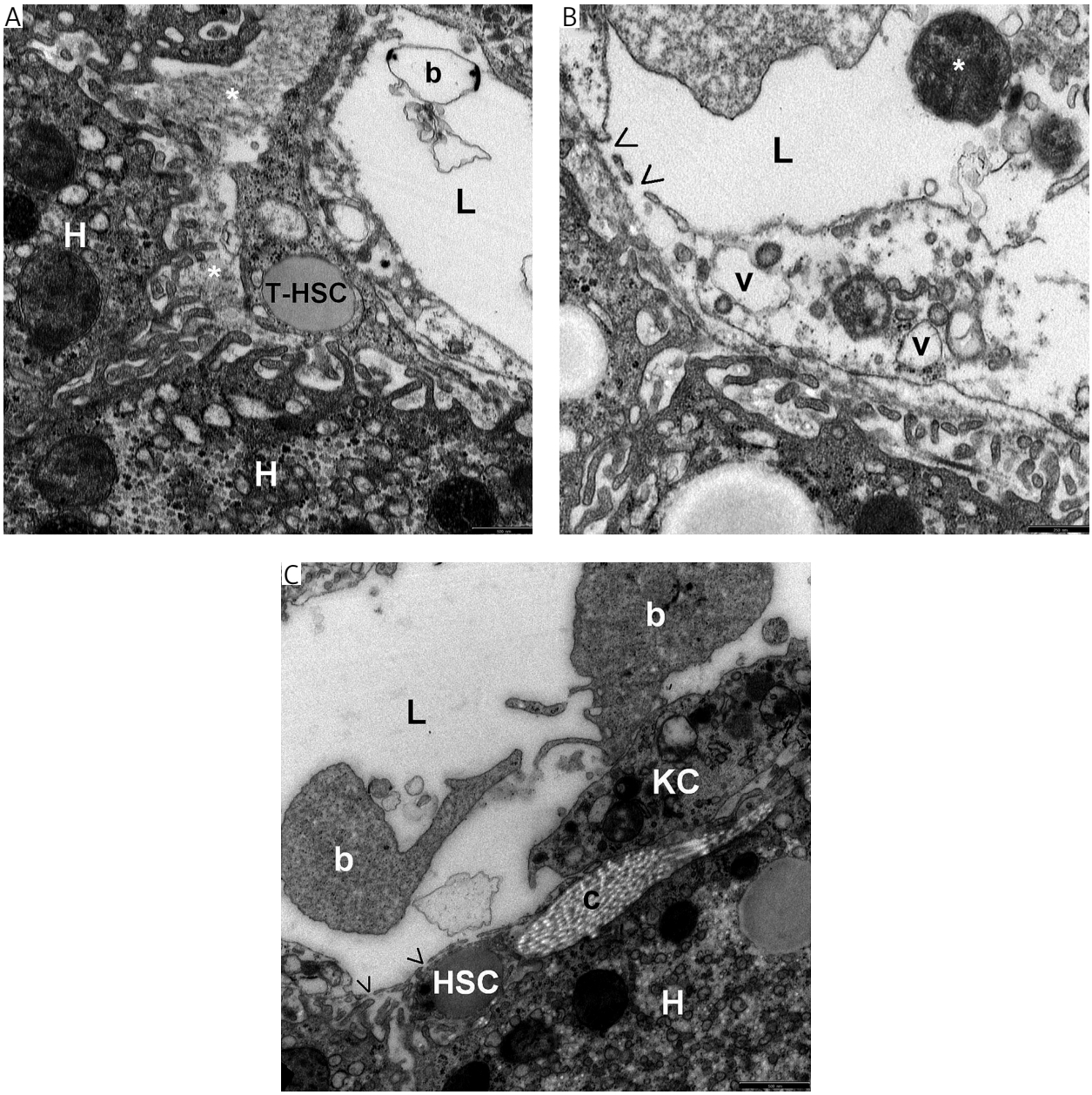


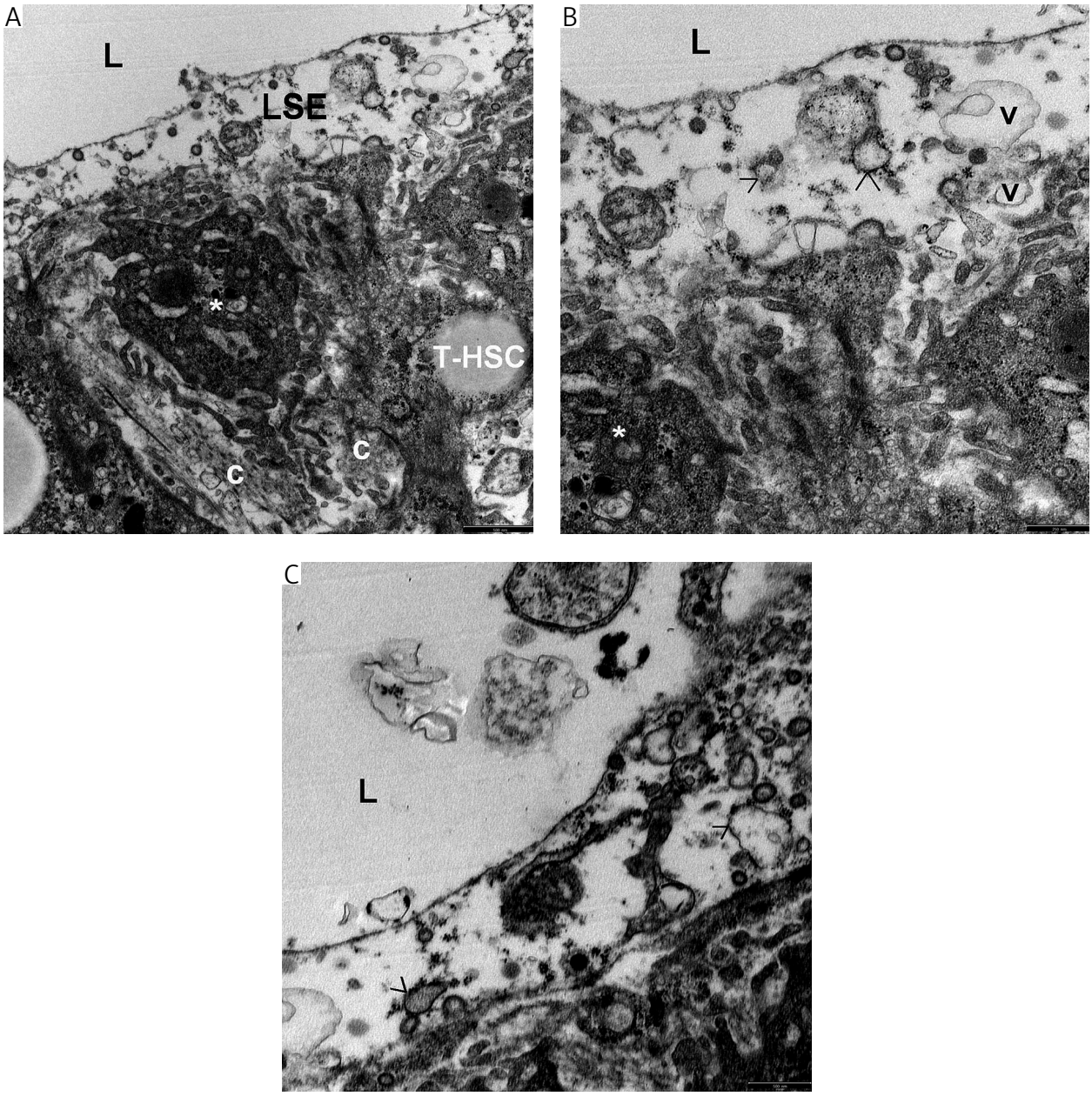
Fig. 1A, B. The ultrastructural picture of activated and distinctly swollen sinusoidal endothelial cells (SEC_1, SEC_2) fragmentarily blocking a patent sinusoidal vessel. SEC_1 located below contains electron-dense phagosomes. The cellular nucleus of SEC_2 has a distinct nucleolus. The cell membrane of both SECs is discontinued in places, and cell organelles fall out to the vascular lumen (L). Focally, the endothelial lining is detached from the sinusoidal wall and the vascular pole of hepatocytes is markedly exposed beneath. The vascular surface of hepatocytes is smoothed. Oligobiopsy material obtained from a child with AIH. Scale bar 2,5 μm (A); scale bar 1 μm (B)

in the presence within their cytoplasm of dark distinct phagolysosomes filled up with absorbed electron-dense material (Fig. 1A, B).

In a number of cases, the cell membrane of damaged sinusoidal endothelium was ruptured and the cell



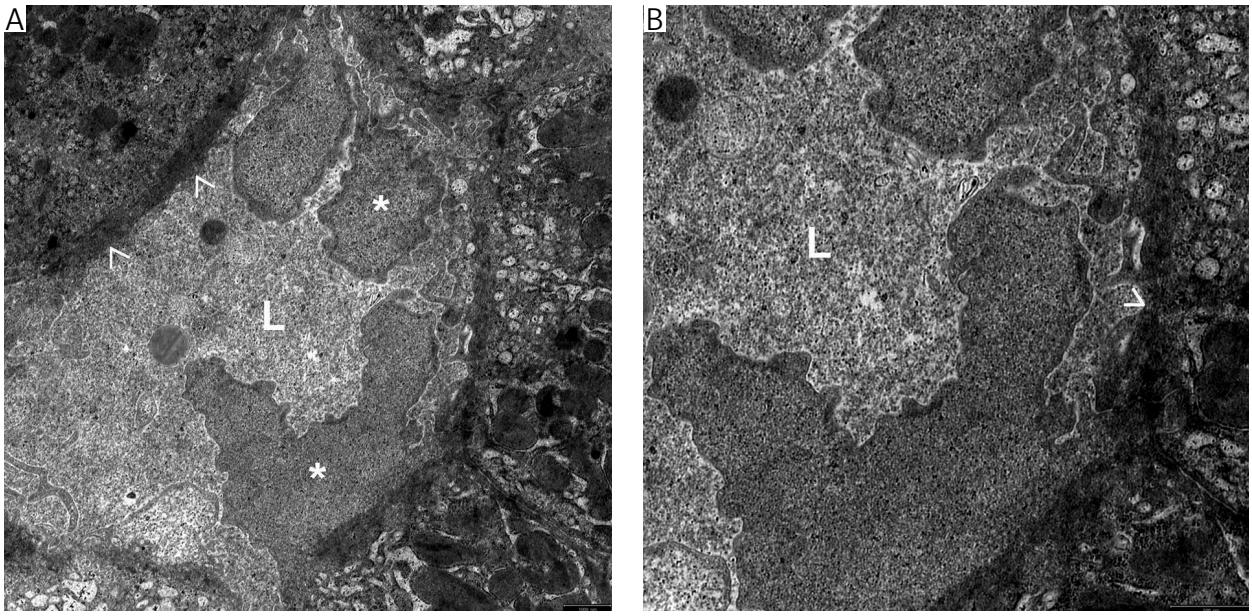
Figs. 2A-C. Electronograms demonstrate variously pronounced changes in liver sinusoidal endothelium (LSE) in oligobiopsy material obtained from children with AIH. A) A fragment of the sinusoidal wall with slightly swollen endothelial lining. The vascular lumen (L) shows blebs (b) – probably a fragment of defatted endothelial lining. Under the endothelial lining, perisinusoidal transformed hepatic stellate (T-HSC) can be seen, surrounded by flocculent, condense extracellular matrix (*), which can be referred to as a morphological precursor of collagen fibers. The surface of hepatocytes (H) directed towards the sinusoidal lumen (L) with microvilli. Scale bar 0.5 μm. B) High magnification of a markedly damaged sinusoidal endothelial cell. Swollen cell membrane, discontinued in places, causes falling out of intracellular organelles, including electron-dense phagosomes (*) and micropinocytic vesicles to the sinusoidal lumen (L). The cytoplasm of the endothelial cell is electron-translucent, shows cistern-like widened ser and ger canals (v), and few dispersed micropinocytic vesicles. Note gaps (>) (region of fenestration). In the upper part of the electronogram, fragment of vascular endothelium with features of necrosis. Scale bar 0.25 μm. C) Fragment of sinusoidal lumen lined with a very thin endothelial lining with adjacent activated Kupffer cell (KC); the endothelial lining shows characteristic "gaps" (>). The sinusoidal lumen exhibits "blebs" (b), with increased electron density that may correspond to dead fragments of endothelial lining. The perisinusoidal space of Disse is markedly extended, contains a hepatic stellate cell (HSC) with the adjacent thick bundle of collagen fibres (c); H – hepatocyte showing proliferating smooth endoplasmic reticulum. Scale bar 0.5 μm



Figs. 3A-C. A) Electronogram demonstrates a damaged liver sinusoidal wall, below which there is a fine „unlaced" fragment of a hepatocyte enclosed by bundles of collagen fibres (c); liver sinusoidal endothelial lining (LSE) in the form of continuous endothelium – markedly swollen, with distinctly reduced number of cell organelles, especially micropinocytic vesicles and with the presence of larger vacuolar spaces. Worthy of note is that LSE has no gaps and basement membrane. A transitional hepatic stellate cell (T-HSC) can be seen in perisinusoidal recess between hepatocytes with the adjacent bundle of collagen fibers. B, C) High magnification of continuous liver sinusoidal endothelium (LSE) showing features of substantial swelling; translucent, extensive devastation of the cytoplasm and not numerous dispersed cell organelles can be seen – dilated ger canals (>), mitochondrium, micropinocytic vesicles accumulating submembranously, cistern-like vacuolar structures (v). No formation of a true basement membrane by LSE can be seen. Oligobiopsy material obtained from a child with AIH. Scale bar 0.5 μm (A); scale bar 0.25 μm (B, C)

contents fell out to the vascular lumen (Figs. 1A, B and 2B). Sometimes the sinusoidal vascular wall was lined with necrotic endothelial cells, which "defatting" to the vascular lumen formed characteristic vesicular blebs of increased electron density (Figs. 2C and 4A, B). Underneath these blebs, the remnants of thinned rudimentary endothelial lining could be seen as well as the exposed sinusoidal plasma membrane of hepatocytes, i.e. the surface of the vascular pole of hepatocytes (Figs. 1A, 2C and 4A, B).

Liver sinusoidal endothelial cell damage was frequently accompanied by significant changes in



Figs 4A, B. The ultrastructural picture of the liver sinusoidal vessel showing marked endothelial cell damage; the sinusoidal vessel lined with necrotic endothelial cells (*), with high electron-dense shadows that bulge into the vascular lumen (L); sites after missing endothelium focally visible (>). The vascular lumen filled up with homogenous microgranular material. Oligobiopsy material obtained from a child with AIH. Scale bar 1μm (A); scale bar 0.5 μm (B)

the population of KCs/MPs. These cells were usually enlarged and showed increased phagocytic activity (Fig. 2C) and damaged mitochondria. We presented the exact ultrastructural picture of Kupffer cells in the same children with AIH in our earlier report [18].

Additionally, the process of fibrogenesis and fibrosis manifested by the presence of flocculent, condense extracellular matrix, which can be referred to as a morphological precursor of collagen fibers (Fig. 2A), and bundles of already mature collagen fibers occupying a considerable part of these spaces (Figs. 2C and 3A) were relatively frequently observed underneath the damaged sinusoidal endothelial lining, i.e. in perisinusoidal spaces of Disse. Collagen fibers adhering directly to hepatic stellate cells (HSCs), especially to the transitional form of HSCs (T-HSCs) were found (Figs. 2A, 2C and 3B). A profound analysis of their ultrastructure will be presented in our future work.

Discussion

The current study is the first to describe the ultrastructural picture of liver sinusoidal endothelial cells in pediatric AIH. We clearly demonstrate variously expressed alterations in the structure of the endothelial lining, from swelling through the so called continuous endothelial cells to their death, which indicates that these cells play a key role in the pathogenesis and progression of this disease. LSEC damage coexisted with significant submicroscopic changes in the population of KCs/MPs, as reported previously [18], and with the process of fibrogenesis, especially in the perisinusoidal spaces of Disse, accompanied by the appearance of T-HSCs. The results of submicroscopic investigations of LSECs were qualitatively similar, although less pronounced, to those observed by Xu *et al*. in adult patients with AIH [9].

Interestingly, even though in approximately half of the analyzed cases of pediatric AIH the sinusoidal vessels were found to undergo transformation of discontinuous LSE, possessing typical fenestrations, into the continuous type of endothelium, without characteristic open pores, we did not observe the formation of a true basement membrane underneath the endothelium in such sinusoids. Thus, we failed to notice distinct morphological transformation of LSECs into vascular-type endothelial cells. It should be taken into consideration that the LSEC defenestration itself and loss of protective properties, as indicated by other authors, is an early event preceding the initiation of perivascular fibrosis [22, 23, 25, 29, 38].

On the other hand, hepatic sinusoidal capillarization characterized by LSE transformation into the continuous vascular type, lack of LSEC fenestration and the formation of an organized basement membrane not only precedes fibrosis, but also promotes HSC activation and fibrosis [22, 23, 29]. Interestingly, the capillarization of sinusoids, commonly observed in cirrhosis, and well described in patients with primary biliary cirrhosis/cholangitis [28, 29, 30, 38] was also reported by Xu *et al*. in adult patients with AIH [9].

It could be assumed that the lack of true basement membrane formation underneath endothelium in pediatric AIH, even when LSECs are markedly damaged, might indicate that regenerative properties of these cells are still preserved and there may be a chance for the lesions to reverse, thus restoring normal endothelial lining. This, however, requires further ultrastructural studies on LSECs and their interactions with other NPCs conducted on broader biopsy material.

Summing up, the current study shows that significant changes in endothelial cell structure, including necrosis and accompanying fibrogenesis, together with other submicroscopic changes, especially in relation to the population of KCs/MPs [17] and HSCs, are markedly involved in the morphogenesis of AIH in children and seem to contribute to the disease progression. The study findings may be used as a comparative material for similar electron-microscopic investigations on the population of NPCs conducted by other research centers concerned with this pathology.

Ultrastructural observations of liver sinusoidal endothelium may also provide a better understanding of the process of fibrogenesis in AIH.

Conclusions

Our results show that severe damage to LSECs, including necrosis and damage to other NPCs, contributes substantially to the morphogenesis of pediatric AIH. It could be assumed that the fact that a true basement membrane is not formed underneath the endothelium, even when LSECs are markedly damaged, might indicate that regenerative properties of these cells are still preserved and there may be a chance for the lesions to retreat.

The authors declare no conflict of interest.

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