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Interrelation between fibrosis, angiogenesis and ductular reaction in progression of chronic steatohepatitis (alcoholic and non-alcoholic) and hepatitis C virus infection

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Key words: *Liver Disease, Hepatitis C, Liver Cirrhosis.*

Most specific morphologic signs of chronic hepatitis were studied at early stages of the diseases.

Aim. To elucidate the interrelation between fibrosis, angiogenesis and ductular reaction in chronic steatohepatitis and hepatitis C viral infection at the stage of cirrhotic transformation.

Methods and results. 45 patients with alcoholic steatohepatitis (ASH), nonalcoholic steatohepatitis (NASH) and hepatitis C viral infection (HCV) at the stage of cirrhotic transformation were enrolled in this study and underwent clinicopathologic examination. The measures of fibrogenesis, angiogenesis and regeneration employed were α -SMA, CD34 and CK19 immunopositive cells respectively. The correlations of these factors with ASH, NASH and HCV were elucidated. Significant ductular reaction was observed only in HCV, whereas low CK19 index could be observed in ASH and NASH. CK19 index in HCV positively correlated with septal fibrosis and angiogenesis. The hepatic neovascularization is proportional to the degree of liver fibrosis in all three diseases.

Conclusion. The results indicate that ductular reaction plays an important role in HCV progression at the stage of cirrhotic transformation whereas fibrosis and angiogenesis prevail in ASH and NASH at the stage of cirrhotic transformation.

Взаємозв'язок між фіброзом, ангиогенезом і дуктулярною реакцією при прогресуванні хронічного стеатогепатиту (алкогольного та неалкогольного) та вірусного гепатиту С

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Більшість специфічних морфологічних ознак хронічних гепатитів вивчені на ранніх стадіях захворювань. Мета роботи полягала в оцінюванні взаємозв'язків між фіброзом, ангиогенезом і дуктулярною реакцією при хронічному стеатогепатиті та вірусному гепатиті С на стадії циротичної трансформації. Здійснили клініко-морфологічне дослідження 45 автопсійних випадків алкогольного стеатогепатиту, неалкогольного стеатогепатиту і вірусного гепатиту С на стадії циротичної трансформації. Кореляції фіброгенезу, ангиогенезу та дуктулярної реакції оцінювали на основі визначення α -SMA, CD34 і CK19 позитивних клітин із наступним морфометричним аналізом у групах алкогольного, неалкогольного стеатогепатиту і вірусного гепатиту С. Виражену дуктулярну реакцію спостерігали при вірусному гепатиті С, а експресія CK19 при алкогольному та неалкогольному стеатогепатиті була низькою. При вірусному гепатиті С цей показник позитивно корелював із септальним фіброзом та ангиогенезом. Показники неоваскуляризації тканини печінки були прямо пропорційні ступеневі фіброзу при усіх трьох захворюваннях. Результати свідчать про важливість дуктулярної реакції у прогресуванні вірусного гепатиту С у порівнянні з механізмами циротичної трансформації при алкогольному та неалкогольному стеатогепатиті, коли домінують процеси фіброгенезу та ангиогенезу.

Ключові слова: печінки хвороби, вірусний гепатит С, фіброз, цироз печінки.

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Взаимосвязь между фиброзом, ангиогенезом и дуктулярной реакцией при прогрессировании хронического стеатогепатита (алкогольного и неалкогольного) и вирусного гепатита С

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Большая часть специфических морфологических признаков хронических гепатитов изучены на ранних стадиях заболеваний. Целью работы была оценка взаимосвязей между фиброзом, ангиогенезом и дуктулярной реакцией при хроническом стеатогепатите и вирусном гепатите С на стадии цирротической трансформации. Проведено клинико-морфологическое исследование 45 аутопсийных случаев алкогольного стеатогепатита, неалкогольного стеатогепатита и вирусного гепатита С на стадии цирротической трансформации. Корреляцию фиброгенеза, ангиогенеза и дуктулярной реакции оценивали на основе определения α -SMA, CD34 и CK19-положительных клеток с последующим морфометрическим анализом в группах алкогольного, неалкогольного стеатогепатита и вирусного гепатита С. Выявленную дуктулярную реакцию наблюдали при вирусном гепатите С, а экспрессия CK19 при алкогольном и неалкогольном стеатогепатите была низкой. При вирусном гепатите С этот показатель положительно коррелировал с септальным фиброзом и ангиогенезом. Показатели неоваскуляризации ткани печени прямо пропорциональны степени фиброза при всех трех заболеваниях. Результаты свидетельствуют о важности дуктулярной реакции в прогрессировании вирусного гепатита С по сравнению с механизмами цирротической трансформации при алкогольном и неалкогольном стеатогепатите, при которых преобладают процессы фиброгенеза и ангиогенеза.

Ключевые слова: печени болезни, цирроз печени, вирусный гепатит С.

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Chronic liver diseases and their end-stages, cirrhosis and hepatocellular carcinoma are leading causes of morbidity and mortality worldwide with enormous socio-economic costs [1]. The main etiologies of liver cirrhosis are chronic hepatitis C virus (HCV) and hepatitis B virus infections, alcohol abuse and as a result of metabolic syndrome reaching epidemic proportions, an increasing prevalence of non-alcoholic steatohepatitis (NASH).

Most specific morphologic signs of chronic hepatitis were studied at early stages of the diseases. Histologic changes typical for variants of cirrhotic transformation in

ASH, NASH and HCV remain unknown. Progression of the diseases depends on persistent chronic injury, associated with accumulation of matrix proteins, microvasculature reorganization and reparative regeneration. All this events are components of the so-called cirrhotic transformation of the liver. Excess deposition of extracellular matrix components including collagen results in the formation of connective tissue septas, capillarization of sinusoids and following reorganization of hepatic lobule. The key cellular mediator of fibrosis is the myofibroblast, which may be generated from a variety of sources including resident mesenchymal cells,

epithelial cells in the process termed epithelial-mesenchymal transition, as well as from circulating fibroblast-like cells derived from bone-marrow stem cells. After the activation myofibroblast serves as a primary collagen-producing cell [2]. Vascular changes are important features of cirrhotic transformation. They include signs of neovascularization and structural changes induced by functional abnormalities (arterialization due to shunting of the blood). Angiogenesis in liver has specific features [3]. Another important morphologic sign, seen in cirrhotic transformation is ductular reaction. Pathogenetically it may be a result of several processes: proliferation of preexisting cholangiocytes, metaplasia of hepatocytes and differentiation of bone-marrow derived stem cells or partially committed intrahepatic precursors (intermediate hepatobiliary cells). In the last variant ductular reaction may be interpreted as indirect sign of reparative regeneration, because immature precursor cells may differentiate into hepatocytes and biliary epitheliocytes [4].

Multiple intercellular and cell-matrix interactions are involved in fibrogenesis, angiogenesis and regeneration but their integrity and consequence are not well understood. Many previous studies conducted to determine molecular processes associated with fibrosis and angiogenesis were performed independently, both in human and experimental models [2]. Although later it was shown that these processes developed synergistically, until now in most researches fibrosis and angiogenesis were studied separately [5]. Recent studies have suggested that the increased ductular reaction has an important role in fibrosis due to transformation of epithelial cells into collagen-producing myofibroblasts and the secretion of specific mediators. However no study has been conducted as yet to examine the interaction between ductular reaction and fibrosis [6,7].

Another important problem is that fibrosis, angiogenesis and regeneration were studied in single diseases: in NASH, in hepatitis C virus infection [6]. Ductular reaction was described mostly in cholangiopathies or other lesions, associated with cholestasis [7].

In the current study we elucidated the possible correlation between fibrosis, angiogenesis and ductular reaction in chronic steatohepatitis and hepatitis C viral infection at the stage of cirrhotic transformation.

Material and methods

45 autopsies from Lviv regional pathologoanatomic bureau obtained between 2007 and 2011 were included in the study. The patients' diagnoses were alcoholic steatohepatitis, nonalcoholic steatohepatitis and hepatitis C virus infection at the stage of cirrhotic transformation.

Diagnosis of ASH was based on the data of alcohol abuse and morphologic signs of alcoholic disease – cardiomyopathy, chronic pancreatitis, alcoholic encephalopathy and typical liver changes. Viral genesis was proved by serological study (RNA HCV) and morphologic signs of HCV (METAVIR criteria) [8]. Diagnosis of NASH was verified by the features of metabolic syndrome and hepatic changes (Brunt criteria) [9].

The study was performed in accordance with the principles of the 1983 Declaration of Helsinki. All procedures were approved by the Danilo Halitsky Lviv National Medical University Ethics Committee (approval number 2/25.02.2008).

Liver specimens were fixed in neutral-buffered formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin, for morphologic evaluation and Masson's trichrome method for fibrosis.

Immunohistochemical stainings were performed on 4µm thick paraffin embedded sections. After standard deparaffinisation procedure primary antibodies were applied. For revealing activated hepatic stellate cells/fibroblasts monoclonal antibodies against α isoform of smooth muscle cell actin (α -SMA) Moa-Hu Alpha Smooth Muscle Actin, Clone 1A4 («DakoCytomation», Denmark) were used. For the examination of endothelial cells sections were processed with monoclonal antibodies Moa-Hu CD34 Class II, Clone QB End 10 («DakoCytomation», Denmark). Cells with the "biliary phenotype" were marked with monoclonal antibodies Moa-Hu Cytokeratin 19, Clone RCK108 («DakoCytomation», Denmark). Control study was performed for every marker in order to rule out false-positive or false-negative results. The endogenous peroxidase activity of the deparaffinized sections was blocked by incubating in 3% hydrogen peroxide solution for 10 minutes. The sections were boiled in citrate buffer for 35 minutes and left to cool before studying with antibodies except CD34. For CD34 the sections were incubated in trypsin for 15 minutes. Then they were incubated with biotin-added anti-immunoglobulin and streptavidin-peroxidase conjugate for 10 minutes. A kit (En-Vision (DakoCytomation) including 3,3'-diaminobenzidine (DAB) was used as the staining agent. Finally, the sections were stained with Mayer's hematoxyline for 60 seconds. All sections were washed with pH 7.6 phosphate buffer in every step up to the DAB application and washed with distilled water after the DAB. All procedures were performed at room temperature.

The results were evaluated under the Leica DM 750/4 (Leica, Germany, 2008) light microscope. Cellular staining was accepted as positive if more than 20% of cells were positive.

The morphometric analysis has been performed at two magnification levels by using computerized image analyzer which included a light microscope Leica DM 750/4 (Germany), digital camera Leica DFC 420 (Germany), and Pentium 200-MHz IBM-compatible computer (RAM, 64 megabytes). The system was programmed by Leica Application Suit (Version 3.8).

The colored microscopic images were saved serially in the memory of a computer and then quantitative examinations were carried out. The principle behind the computer-based morphometry is a different staining pattern of cells following immunohistochemistry. The cellular cytoplasm/membrane becomes brown. For the analysis, 20 photographs of random low-power fields (100 magnifications) and 20 high-power fields (400 magnifications) were taken of each liver sample. Large bile ducts and vessels were excluded. Photographs were stored as 1280 X 1024 pixel RGB-bitmaps (bmp) with a color-resolution of 24 bits per pixel.

Quantification was performed by assessing the ratio of stained tissue to the total area of the liver section analyzed using Image-ProPlus (Version 6.) imaging software. The indexes were calculated in the three compartments: general, septal and lobular.

Statistical evaluation: The results of the study were analyzed by "STATISTICA FOR WINDOWS 6.0" (Statsoft,

USA) package software. The data were expressed as mean value ± standard deviation (M±SD). The differences between groups were analyzed using ANOVA analysis (LSD test). The correlation between two parameters was tested by Spearman rank correlation matrix. The results were evaluated at the p<0.05 significance level.

Results

The clinical features of the groups are shown in table 1. Most of the clinical features in ASH, NASH and HCV patients were not significantly different.

Table 1

Characteristic features of patients with alcoholic steatohepatitis, nonalcoholic steatohepatitis and hepatitis C virus infection (minimal, maximal, and mean value)

Parameter	Alcoholic steatohepatitis (n=15)	Nonalcoholic steatohepatitis (n=15)	Hepatitis C virus infection (n=15)
Gender (M/F)	11/4	5/10	11/4
Age	41-65 (55,7)	52-71 (60,6)	22-60 (43,6)
Total bilirubin (μmol/l)	41,6-324,5 (138,7)	11,9-270,3 (125,9)	37,8-419,4 (141,7)
Alanine aminotransferase (mckat/L)	0,3-24,2 (7,11)	0,43-22,5 (8,08)	0,2-20,3 (4,8)
Prothrombin index (%)	32-71 (51,2)	47-93 (72,4)	42-77 (59,3)
Fibrosis stage (METAVIR) (F2,3 - n)	F2 - 5 F3 - 10	F2 - 12 F3 - 3	F2 - 4 F3 - 11

NB: M – male, F – female.

The comparison of the α-SMA+ cells areas showed significant differences in all three groups (fig. 1). Septal α-SMA was the highest in HCV and the lowest — in NASH. The opposite results were revealed in the analysis of lobular α-SMA: index was the highest in the group with NASH, lower in ASH and the lowest in HCV. Such opposite tendencies explained relative “equalizing” of the general α-SMA indexes. However, general α-SMA was the highest in HCV, and the lowest — in NASH with significant differences between all groups (p< 0.001).

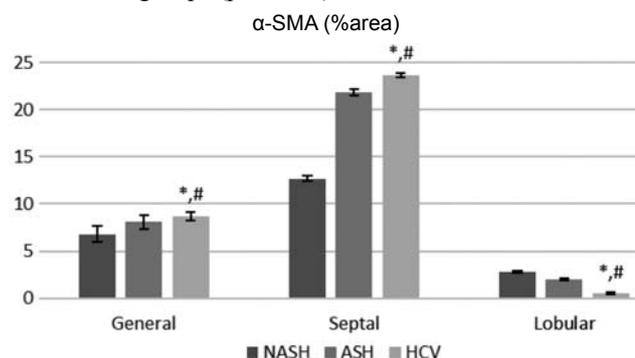


Fig. 1. Analysis of α-SMA in NASH, ASH and HCV. NB: * p<0.05 vs. NASH group; # p<0.05 vs. ASH group.

The study of the CD34+ cells area (fig. 2) showed that general index was the highest in HCV, mostly due to the level of the septal index which was severely higher than in other two groups. Lobular CD34 was the highest in NASH and the lowest in HCV. The group of ASH had the intermediate level (significant difference with other two groups, p<0.01).

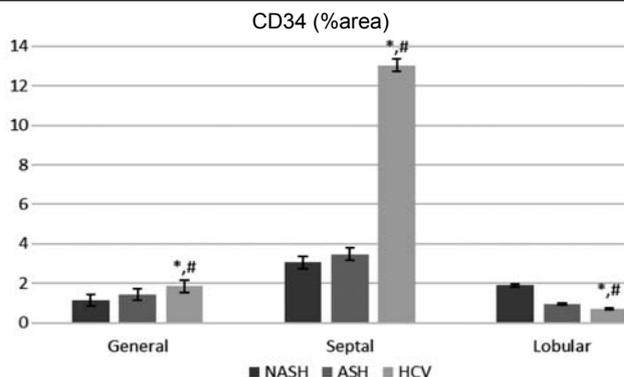


Fig. 2. Analysis of CD34 in NASH, ASH and HCV. NB: * p<0.05 vs. NASH group; # p<0.05 vs. ASH group.

The evaluation of CK19-positive cells area in the liver section (fig. 3) showed the highest degree in HCV group. Differences of this marker from the markers revealed in NASH and ASH were statistically significant. In addition to quantitative parameters localization of ductular reaction and cellular phenotype were analyzed. Diffuse spreading of the cells with «biliary phenotype» within the thickness of connective tissue septas was found in NASH and ASH. Most ductules were laid by homogenous cuboidal cells and contained distinct lumens. In HCV CK19-positive cells were revealed mostly in the site of marginal plate between the lobular parenchyma and connective tissue septa. In histologic examination cellular population with «biliary phenotype» was more heterogenous and some ductules had no lumens.

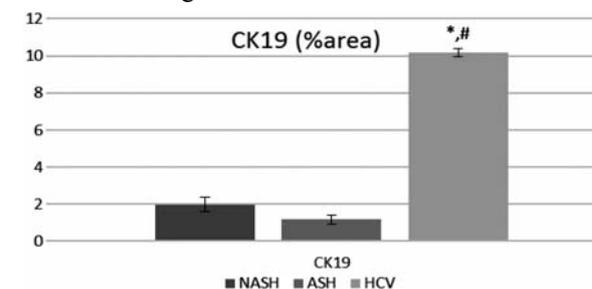


Fig. 3. Analysis of CK19 in NASH, ASH and HCV. NB: * p<0.05 vs. NASH group; # p<0.05 vs. ASH group.

The correlations between the endothelial marker and fibrogenesis index were studied. Positive correlations were found for the septal CD34 and septal α-SMA (r=0.507, p<0.001). The lobular expression of CD34 correlated with marker of lobular fibrosis (correlation of lobular CD34 and α-SMA; r=0.79, p<0.001).

In order to evaluate the role of CK19+ cells in the morphogenesis of fibrosis correlations between this marker and indexes of fibrogenesis and angiogenesis were studied. Direct significant correlation was revealed between the CK19 and values of septal (α-SMAS: r=0,6899, p=0,001; CD34S: r=0,2878, p=0,055) and general indexes (α-SMAG: r=0,6257, p<0,001, CD34G: r=0,6428, p<0,001). Inverse correlation was found between the CK19 and lobular indexes of fibrogenesis and angiogenesis (α-SMAP: r= -0,9513, p<0,001; CD34P: r= -0,7003, p<0,001).

Fibrogenesis depends on the cells which are able to produce elements of the extracellular matrix – «myofibroblasts». The heterogeneous population of fibrogenic cells in the liver is presented by portal/septal fibroblasts, hepatic

stellate cells and cells with intermediate phenotype. The activation of these cells is associated with the changes in the phenotype (expression of α -SMA and other markers) and functional activity (increased contractility, proliferation, secretion, migration). The quantitative analysis of the α -SMA + cells area at the stage of cirrhotic transformation in chronic steatohepatitis and viral hepatitis shows the highest level of septal fibrosis in HCV and prominent intralobular fibrogenesis in NASH. These findings coincide with results of other researches [2].

Recent studies have revealed that angiogenesis plays an important role in many pathological events, including liver fibrosis development [11]. Angiogenesis in the liver is manifested by septal neovascularization and phenotypic changes of the hepatic sinusoidal endothelial cells. Vascular marker CD34 is expressed in the endotheliocytes of portal vessels, central vein and some peripheral sinusoids. It has been reported that CD34 is not expressed by healthy SECs, but when SECs alter their phenotype, they can express CD34 [3]. Our results show increased level of CD34+ cells area in ASH, NASH and HCV at the stage of cirrhotic transformation. The highest degree of angiogenesis, mostly in the septal compartment, is seen in HCV. Intralobular CD34+ cells area is prominent in NASH. A recent study on chronic hepatitis C has shown that the number of CD34+ new vessels was significantly increased and positively correlated with the fibrosis stage [10]. The degree of CD34+ neovascularization was almost parallel to the development of liver fibrosis in NASH [10]. These results are consistent with our findings: presence of positive correlation between the markers of fibrogenesis and angiogenesis in all three diseases.

Ductular reaction previously was interpreted only as a sign of cholestatic liver diseases. Recently it was shown that proliferation and differentiation of cells with «biliary phenotype» are associated with progressive fibrosis. Two mechanisms may explain this phenomenon. The first named epithelial-mesenchymal transition suggests transformation of liver epithelial cells (hepatocytes and cholangiocytes) towards myofibroblasts. EMT markers (fibroblast specific protein-1, vimentin) were shown to localize in DR elements in liver in primary biliary cirrhosis, alcoholic liver injury, NASH and HCV [11]. The second mechanism is based on the secretory properties of the activated progenitor cells which are able to produce inflammatory cytokines, chemoattractive proteins and inhibitors of apoptosis [7] with profibrogenic

effect. Our results show the most prominent ductular reaction in HCV. Multiple groups of CK19+ cells are revealed in the marginal zone between the stromal and parenchymal compartments. While in ASH and NASH new-formed ductules are not so prominent and have a diffuse distribution in the connective tissue septa. These observations are in harmony with the findings of Italian researches who observed prominent ductular reaction in HCV [5].

Analysis of cellular phenotype in CK19+ cells showed differences between the etiopathogenetic groups. In ASH and NASH homogenous cuboidal cells and distinct ductular lumens likely were the result of biliary epithelial cell proliferation from the preexisting ductules. In HCV genesis of heterogenous cellular population with «biliary phenotype» and ductules with indistinct lumens may be associated with activation and proliferation of immature precursors – intermediate hepato-biliary cells. Such histologic findings may be found in routine HE staining and used as indirect criteria of predominant mechanism of ductular reaction.

The presence of direct correlation between the septal α -SMA, CD34 and CK19 confirms importance of ductular reaction in the progression of fibrosis in HCV likely due to exertion of profibrogenic activity by CK19+ cells. In ASH and NASH such associations are not so evident. Presence of inverse correlation between CK19 and lobular α -SMA, CD34 suggests evidence of the other mechanisms maintaining progressive fibrogenesis and angiogenesis in the lobular compartment (oxidative stress, repair). Direct correlation between the CK19 and septal markers in these diseases likely confirms coexistence of different mechanisms of fibrosis at the stage of cirrhotic transformation.

Conclusions

1. At the stage of cirrhotic transformation only the liver of HCV cases had marked ductular reaction, whereas in ASH and NASH livers did not.
2. CK19 index in HCV positively correlated with septal fibrosis and angiogenesis.
3. The hepatic neovascularization is proportional to the degree of liver fibrosis in all three diseases.

Perspectives for future studies. The results indicate that the interrelation between α -SMA, CD34 and CK19 may play important role in the progression of HCV. More researches are necessary for understanding of the mechanisms of cirrhotic transformation in ASH and NASH.

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