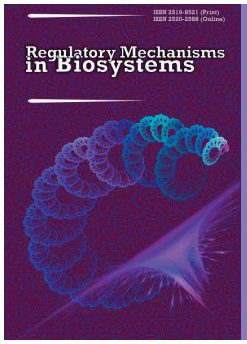

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Levels of isoforms of fibronectin and α_5/CD49e integrin on lymphocytes and in blood plasma in the conditions of chronic diffuse liver diseases

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Chronic diffuse liver diseases are characterized by accumulation of complex inflammatory infiltrate in the liver tissues, blood, and lympha, and activation of the immune system. Leukocytes become involved in the area of inflammation after the activation of receptors of blood adhesia, particularly integrins and their ligands. Plasma lymphocytes quickly activate the function of integrins by changing their conformation, leading to high affinity and underlying the formation of strong stable connection between the components of extracellular matrix. A vitally important role in the process of liver fibrogenesis is performed by a pro-fibrogenicic protein fibronectin which induces the expresson of collagen genes and precedes the deposition of other components of matrix. The studies were conducted in the group of patients suffering from chronic diffuse liver diseases of non-viral etiology aged 28–60 years, n = 36 and in the group of 15 practically healthy volunteer donors aged 25 to 52 years without a history of liver diseases using the methods of flow cytofluorometry, immunoenzymatic analysis, and quantitative real-time polymerase chain reaction. The patients of the group with chronic diffuse liver diseases were observed to have statistically significant decrease in the concentration of plasmatic form of fibronectin measuring 27.6% compared with the control group. We determined increase in the concentration of cellular fibronectin in blood plasma of patients with the diseases on average accounting for 63.8% compared with the norm, and the highest increase in this parameter equaling 77.2% was seen in patients suffering from drug-induced hepatitis. Significant increase in the level of exposure of cellular FN on blood lymphocytes was determined in patients with chronic diffuse liver diseases, measuring 231.8%, whereas the level of plasmatic form of fibronectin in these cells was decreased (statistically unreliable). For α5-integrin subunit, we determined a 390.8% increase in the level of its exposure in blood lymphocytes in the surveyed groups compared with the control. Level of blood lymphocytes that express the cellular fibronectin significantly decreased by 140.1%. Statistical characteristics of diagnostic possibility of the parameters of the level of plasmatic and cellular fibronectin in blood, determined over the analysis of ROC-curves, demonstrated excellent informativeness of these tests. Analysis of the possibility of predicting the presence of pathology using the model of logistic regression revealed zero error of prediction and maximum efficiency of the tests: intensity of exposure of α_5-integrin receptor on the surface of lymphocytes, intensity of exposure of plasmatic fibronectin on the surface of lymphocytes, intensity of exposure of cellular fibronectin on the surface of lymphocytes, concentration of plasmatic fibronectin in blood, concentration of cellular fibronectin in blood plasma. These parameters may be proposed for further surveys for developing serologic biomarkers based on the parameters for diagnostics of chronic diffuse liver diseases.

Keywords: plasmatic fibronectin; cellular fibronectin; α_5-integrin cellular; ITGA expression; lymphocytes.

Introduction

The most important physiological event during inflammation of the liver is activation of the intrinsic immune system with involvement of plasma leukocytes from the blood flow, their constant migration and fast accumulation in the damaged areas and areas of infection. Neutrophil granulocytes are the commonest leukocytes in the blood flow, first appearing in the damaged or traumatized areas at the early stages of liver inflammation (Koh & DiPietro, 2011). Much less numerous monocytes are activated by interferons produced by T-cells and organize protective structures – granulomes – around antigen. After extravasation, monocytes can differentiate into Kupffer cells of the liver – specialized macrophages which are a part of the reticular endothelial system (Burns et al., 2001). Lymphocytes, unlike granulocytes and monocytes, can recirculate many times over their life: they migrate from blood to the tissue, enter the lymphatic system, percolating the lymph nodes and return to the blood flow. In response to the inflammatory irritators, lymphocytes may accumulate in the damaged areas (Springer, 1995) and directly affect the formation of scars and fibrosis (Koh & DiPietro, 2011).

Accumulation of interstitial leukocytes in the areas of inflammation is preceded by the activation of cellular adhesion receptors such as selectins, integrins and their ligands. Since selectins provide initial binding of leukocytes to the endothelium of the vessels and their removal from the blood flow, the integrins underlie transendothelium migration due to additional interrelations with extracellular matrix and instant changes in the degree of their adhesivity (Burns et al., 2001). Integrin heterodimer molecules transduct bidirectional signals, necessary for fast reaction of cell to change in the environment, through the plasmatic membrane (Hu & Luo, 2013). Leukocytes which circulate the blood flow support their integrins in non-active conformation to avoid contact with undamaged walls of the vessels, but in the conditions of inflammatory processes integrins quickly activate to provide migration to damaged regions (Barreiro & Sánchez-Madrid, 2009). Therefore, decreased level of these molecules on the surface of leukocytes hinders their fast and efficient flow to the areas of inflammation

without damage to the integrity of endothelial barrier (Springer, 1995). Two-four weeks after antigen stimulation, $\alpha_5\beta_1$-containing integrins appear on lymphocytes. They interact with ligands of extracellular matrix: laminin, collagen and fibronectin, providing migration and accumulation of interstitial leukocytes during inflammation (André et al., 2010).

Fibronectin (FN) is a glycoprotein of extracellular matrix which plays a vitally important role during restoration of the liver tissue. Plasmatic fibronectin (pFN) is produced by hepatocytes, circulates in plasma in non-active form, and during tissue damage becomes involved in the fibrin clots, taking effect on the function of thrombocytes and underlying hemostasis. Cellular FN (cFN) is synthesized by many types of cells, including fibroblasts, epithelial cells, endothelial cells, leukocytes, etc. It is collected by cells when they migrate for restoration of the damaged tissue, contributes to the support of the carcase of the extracellular structure by active binding with other matrix proteins. Both forms of FN differently express in time: plasmatic FN circulates in blood constantly and functions at the early stages of inflammation, whereas cellular FN expresses and accumulates locally in the zones of active morphogenesis and remodeling of the tissue (Koivisto et al., 2014).

Plasmatic and cellular fibronectins exhibit mutual general structural organization and immunologic identity. However, unlike the cellular FN, plasmatic FN lacks EDA and EDB domains and only one of its subunits has V-region. Change in the structure of cellular FN leads to changes in kinetic abilities to polymerase into fibril, creating matrix, and increased expression in cellular fibronectin is distinctive for fibrolithic impairments. Though the commonest component of fibrosis tissue is collagen, its sedimentation is possible only over formation of fibronectin matrix (Dolgikh et al., 2018). During fibrosis, there occurs notable expression of isoforms of cellular FN, particularly EDA, EDB and oncofetal IIICS (To & Midwood, 2011). Leukocytes are known to bind with fibronectin using integrins both due to RGD-sequence and RGD-dependent way. However, these integrins support fibrillogenesis of fibronectin: there is a presumption that only $\alpha_5\beta_1$ (VLA-5) integrin takes part over invasion of cells of connective tissue. It is selective only in relation to fibronectin, demonstrates abilities to efficiently bind with its compact soluble dimmers and assemble fibrillar matrix. Such interactions provide important data for contractile organization of cytoskeleton of cells, contribute to adhesion, migration, survival, proliferation of cells and remodeling of connective tissue (Huveneers et al., 2008).

Through RGD motif and $\alpha_5\beta_1$, cellular FN activates transmission of signals for cellular production of matrix metalloproteinases. They take part in events of remodelling of extracellular matrix and produce FN fragments which could additionally stimulate behaviour of cells (Aziz-Seible & Casey, 2011). Fragmentation of FN is underlain by hydrolysis of flexible connections in the areas of linkage between functional domains of glycoprotein. Therefore, N-terminal fragment 70 kDa which contains modules of I type and gelatin-binding region, binds in RGD-dependent way with cellular surface and with fibrils of native FN with high affinity and takes part in creation of matrix. Moreover, studies revealed that when contacting native FN over at first links to the cellular surface particularly through 70-kDa area of N-terminal domain. This causes conformational changes in the integral FN molecule and uncovers sites, including RGD, which take part in future interactions (Tomasini-Johansson et al., 2006).

Chronic diffuse liver diseases (CDLD) are accompanied by gradual replacement of parenchyma of the organ of scar tissue. The extent of damage is reflected in the corresponding change in the structure of the liver, vessel architecture, basement membrane-like matrix within the space of Disse, chronic damage to hepatocytes, activation of perisinusoidal cells, etc. At the same time, there occurs release of anti-inflammatory, profibrogenic and promitogenic stimuli, after response to which the liver extracellular matrix (ECM) starts to be gradually reconstructed by fibril-forming collagen of I and III types and fibronectin (Lee & Fridman, 2011). At the beginning CDLD manifest as hepatic steatosis characterized by increased deposition of fat in liver cells and increase of the organ. Steatosis can progress to hepatitis, the condition indicated by clearly manifested inflammatory changes. Constant damage causes development of scar tissue which ultimately can replace functional tissue of the liver, lead to liver failure, fibrosis and cirrhosis. All pathological conditions include inflammatory reaction including healing mechanisms related to expression by Kupffer cells, and also cells of the immune system of cellular FN. Cellular FN activates perisinusoidal cells, increases their survivability and leads to transition of these cells to miofibroblast phenotype through regulated TGF-β_1 mechanism induced by EDA. In turn, activated perisinusoidal cells are the main sources of intense production of the constituents of the connective scar tissue (Aziz-Seible & Casey, 2011). Therefore, leukocytes of the immune system responding to damage of the liver, using a number of complex signal mechanisms, can initiate significant increase in the expression of cellular fibronectin, triggering processes that lead to accumulation of fibrosis matrix in the damaged organ.

The objective of the study was determining the diagnostic and predictive efficiency of the parameters of concentration of fibronectin in blood plasma; level of exposure of pFN, cFN and $\alpha_5\beta_1$ integrin receptor on lymphocytes; amount of lymphocytes, out of their total amount, which expose pFN, cFN and $\alpha_5\beta_1$ over chronic diffuse liver diseases, namely non-alcohol fatty liver disease (steatosis), steatohepatitis, alcoholic liver disease, drug-induced hepatitis.

Materials and methods

The research was carried out following the bioethical norms according to the regulations of WHO, Helsinki Declaration of General Assembly of World Medical Association (1989), Convention of the European Council on Human Rights and Biomedicine (1977), Council for International Organizations of Medical Sciences, International Code of Medical Ethics (1983), current legislation of Ukraine, as confirmed by the Committee of Bioethics of the Dnipropetrovsk Medical Academy of the Ministry of Healthcare of Ukraine. Informed consent was received from all the subjects of blood withdrawal. Blood was withdrawn in the morning at the same time from the intermediate basilic vein of patients on an empty stomach in the amount of 15 mL. For this purpose, we used disposable test tubes with anticoagulants K_2EDTA and K_3EDTA,the colour of the cap of test tubes was violet. In the same way, blood samples were withdrawn from healthy donors.

We surveyed blood from patients suffering from chronic diffuse liver diseases aged 28–60 years who were at the treatment hospital in the Department of the Liver and Pancreas Diseases at the Institute of Gastroenterology of the National Academy of Medical Sciences of Ukraine, particularly 36 patients with CLD, including: patients with non-alcoholic fatty liver disease steatohepatitis (NAFLD) n = 12, patients with steatohepatitis (SH) n = 13, patients with drug-induced hepatitis (DISH) n = 6, patients with alcoholic hepatitis (ASH) n = 5. The control group comprised 15 clinically healthy volunteer donors (PHD) at the age of 25–52 years without history of liver diseases or other immune diseases.

Part of venous blood was centrifuged over 10 min at 3,000 rpm on centrifuge CLMN-P10-01 Elekon (Liston, Russia), isolated plasma was divided into aliquots and kept in –76 °C in Eppendorf test tubes for further analysis of the levels of plasmatic and cellular fibronectins. From the other part of blood, we isolated fraction of lymphocytes for survey of exposure of two forms of fibronectin and subunit of its α_5/CD49e integrin receptor. Fraction of lymphocytes was selected by isolating cells using Ficoll-Paque 1.077 density gradient. For this purpose blood was held for 40–60 min at room temperature until clear separation of erythrocytes and plasma. To prepare lysing solution with density gradient measuring 1.077 g/cm, we used Ficoll-400 (Pharmacia Fine Chemicals, Sweden) and Triombrast (Farmak, Ukraine).

To centrifugation test tubes for density gradient we added a layer of obtained plasma with the upper layer of erythrocytes; ratio of gradient and plasma equaled 1:2. Test tubes were centrifuged in a bucket-rotor with acceleration of 1,500–1,800 rpm over 40 min at a temperature of 20 °C. After separation of plasma and fraction of lymphocytes with inclusions of monocytes, cells were rinsed three times using buffered normal saline (BNS) with subcequent centrifugation over 2 min at 2,400 rpm. After rinsing, we prepared the aliquots of operational concentrations of lymphocytes with BNS (300 thous/mL in each sample). Vitality of the cells (over 90%) was determined using trypan blue (Novikov & Novikova, 1996). Levels of cFN- and pFN-positive lymphocytes were determined using the method of flow cytofluometry with primary antibodies: mouse monoclonal IgG to cell-binding FN fragment (FN30-8; M010 TaKaRa Shuzo

Co. Ltd., Shiga, Japan) and rabbit polyclonal antibodies to FN (ab2413; Abcam, Cambridge, UK), respectively. As secondary antibodies we used fluorescein isothiocyanate (FITC), conjugated goat anti-mouse IgG antibodies (H+L) (A16067; ThermoFisherScientific, US) and goat anti-rabbit IgG antibody, labeled with phycoerythrin – PE (F0609, SantaCruzBiotechnology, USA), respetively. To determine the level of exposure of integrin receptor we used goat polyclonal anti-integrin α_5/CD49e antibodies (AF1864, R&D Systems, US) and respectively donkey anti-rabbit IgG (H+L), labeled with phycoerythrin (PA1-29953; Thermo Fisher Scientific, US). Control of dead cells was made after binding with propidium iodide. The data were recorded on a flow cytofluorimeter Beckman Coulter EPICS (Beckman Coulter, USA, 2001). The density of exposure was calculated according to FCS Express 3 software (De Novo Software, USA, 2001).

Levels of pFN and cFN in blood were determined using the enzyme-linked immunosorbent assay (ELISA) with specific antibodies. The stages of ELISA included: preparation of microplate for adhesion of proteins; incubation of control and experimental samples for 2 h at a temperature of 37oC followed by rinsing in the solution of BNS which contained Tween-80 – 0.05% twin phosphate buffer (TPB); blocking using 1% albumin solution, incubation with primary antibodies for 12 h at the temperature of 37 °C followed by rinsing; incubation with secondary antibodies for 2 h in 37 °C followed by rinsing in TPB. In the case of cellular FN, the monoclonal antibodies provided adhesion with RGD-cell binding domain located in the center of this glycoprotein (FN30-8; M010 TaKaRa Shuzo Co. Ltd., Shiga, Japan), in the case of plasmatic FN – with all binding sites in fibronectin (ab2413; Abcam, Cambridge, UK). Antibodies were diluted in the proportion of 1:5,000 according to the manufacturer's guidelines TPB. To determine the amount of fibronectins bound with primary antibodies, we used secondary antibodies: goat antibodies to mouse IgG, labeled with horseradish peroxidase (HRP) for cellular FN (A16066, Thermo Fisher Scientific, US), diluted in the proportion of 1:4,000 and goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase for plasmatic FN (31466, ThermoFisherScientific, US), diluted in 1:3,000 proportion. For calorimetric determination of absorbtion, into the microplate wells, solution of coloring dihydrochloride o-phenylenediamine (OPD) was added with 9% solution of H_2O_2, the reaction was stopped with 2 N solution of H_2SO_4. Optical density of the examined samples was determined using spectrophotometer for reading Humareader microplates (Human, Germany, 2001) at the wavelength of 492 nm.

Analysis of ITGA5 expression was performed using quantitative real-time polymerase chain reaction (PCR). Isolation of RNA from lymphocytes was carried out using Trizol reagent (Invitrogen, USA). For this purpose, to the suspension of lymphocytes, we added 80 μL of Trizol reagent, mixed, and added 20 μL of chloroform, mixed again and centrifuged at 16,000 g and +4 ºC over 10 min. We selected 50 μL of supernatant and RNA and sedimented them by an even volume of iso-propanol for two hours at –20 ºC. The RNA sediments obtained in centrifugation were rinsed with 100 μL 75% ethanol and diluted it in ribonuclease-free sterile water (QIAGEN, Germany).

To amplify cDNA of ITGA5 (integrin, alpha 5 (fibronectinreceptor, alphapolypeptide), we used the following primers: forward (5'–GTGGTGCTGTCTACCTCTGT –3' and reverse (5'–TCAGTGGCTCCTTCTCTGTG–3'). Nucleotide sequences of these primers correspond to the sequences 346–365 and 576–557 cDNA of ITGA5 of humans (GenBank number NM_002205). Size of amplified fragment equaled 231 bp.

The amount of RNA taken for the analysis was estimated according to the level of expression of beta-actin mRNA (ACTB). Amplification of beta-actin cDNA was conducted using forward – 5'– GGACTTCGAGCAAGAGATGG–3' and reverse – 5'–AGCACTGTGTTGGCGTACAG–3' primers. Nucleotide sequences of these primers correspond to the sequences 747–766 and 980–961 of human cDNA ACTB (GenBank number NM_001101). The size of amplified fragment was 234 bp. Primers were received from Sigma-Aldrich company (USA). To amplify cDNA of rhibosomal protein S16, we used the following primers: forward – 5'–GGCAATGGTCTCATCAAGGT–3' and reverse – 5'–TCTCCTTCTTGGAAGCCTCA–3'. These oligonucleotides correspond to nucleotide sequences 133–152 and 373–354 of published cDNA of human rhibosomal protein S16 (GenBank number X00351). Reverse transcription of RNA was performed using Quantitect ReverseTranscription Kit (QIAGEN) according to the manufacturer`s protocol. For reverse transcription reaction we took 1 μL of RNA and mixture of primers from the kit. Then, we amplified the obtained preparations of cDNA. Reaction mixture contained 8 μL of nuclease-free water, 1 μL of cDNA, 1 μL of 10 mmol/L of the mixture of primers and 10 μL of two-fold mixture for polymerase chain reaction (QIAGEN). Amplification products were analyzed using electrophoresis device in 2% agarose gel, staining DNA with 5x Sight DNA Stain (EUROMEDEA). Gels were analyzed in Quantity One Bio Rad System (USA).

The data were statistically analyzed using R software packs and EasyROC 1.3.1 (GNUGPL 2, USA, 2020). To describe the extent of the central tendency of quantitative features, we used mean arithmetic (x) and standard errors (SE). To assess the differences between the quantitative parameters between the groups, we used the Tukey test with Bonferroni correction. To analyze the interrelation between the parameters we used correlational analysis with consideration of coefficients of Spearman range correlation (ρ). Correlation coefficient $P < 0.01$ indicates strong correlational relationship; $P < 0.05$ – relationship of average strength. The indicator of the level of statistical significance for all species in the analysis accounted for 5%. The differences were considered statistically significant if the probability of exidental occurrence of differences did not exceed 0.05 ($P < 0.05$). To evaluate the efficiency of use of diagnostic markers, we used the method of traditional ROC-analysis (Mandrekar, 2010). ROC-analysis and visualization of ROC-curves were conducted in EasyROC 1.3.1 software pack. The analysis revealed values of statistical parameters of efficiency of diagnostic tests: sensitivity (Se) and specificity (Sp); to characterize the informativeness the determined values of the area under the ROC-curve (AUC – Area Under Curve), we determined the cutoff points for the condition norm-pathology. The values of the area from ROC-curve were interpreted in the parameters of diagnostic accuracy: 0.91–1.00 – excellent, 0.81–0.90 – very good, 0.71–0.80 – good, 0.61–0.70 – average, 0.51–0.60 – unsatisfactory; value of 0.50 corresponds to inapplicability of the model. The cutoff point was calculated using the Youden method. To develop the box-plot graphs we used R software pack for statistical analysis of the data (Lang & Sesik, 2011). To determine the probability of pathological condition and determining optimum combination of tests, we used the model of logistic regression as classifier (Logit Model). To implement the model we used Starts library of R pack. This model shows equally effective results both in the normal distribution of independent variables and in the opposite case. It predicts the possibility of pathology through the use of continuous independent variables, both alone, and with a group of parameters simultaneously.

Results

In the patients with chronic diffusive diseases of the liver, compared with healthy donors, we observed decrease in the level of ITGA5 mRNA expression by 11.0%. Expression of ITGA5 (integrin, alpha 5 (fibronectinreceptor, alphapolypeptide) in blood lymphocytes was determined in relation to the beta-actin mRNA expression as the control gene.

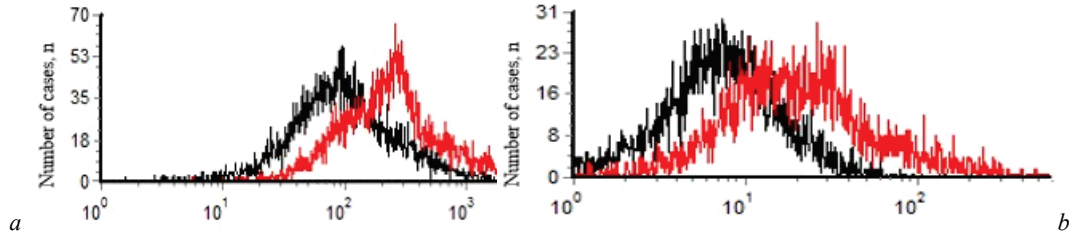
Analysis of density of exposure of fibronectin on lymphocytes demonstrated dependence of their distribution on the type of fibronectin (Table 1). We reliably determined exposure of cellular fibronectin to significantly increase in case of chronic diffuse liver diseases compared with the control group, particularly by 231.8% ($P < 0.001$), whereas insignificant decrease in exposure of plasmatic form of FN exhibited statistically insignificant differences. For α_5-integrin subunit, we determined significant increase in the exposure, measuring 390.8% ($P < 0.001$) on lymphocytes in the presence of chronic diffuse liver diseases (Table 1). We also recorded statistically reliable decrease in the content of cellular FN-expressing lymphocytes, being at the level of 140.1% ($P < 0.001$). Intensity of protein exposure on blood lymphocytes is presented in Figure 1. Patients of all groups with CDLD were observed to have statistically reliable decrease in pFN concentration compared with the control group, equaling 27.6%, including, by group: pFN decreased by 23.5% in the group of patients with steatohepatitis, and by 30.5% in the group of patients with drug-induced hepatitis. Greatest decrease in the concentration measuring 31.5% was seen in the group of patients with alcoholic liver disease (Fig. 2a).

Table 1
Statistical characteristics of exposure of plasmatic fibronectin, cellular fibronectin, α_5-subynit of integrin receptor on lymphocytes of blood plasma from patients suffering from chronic diffuse liver diseases and the amount of lymphocytes which expose the surveyed proteins (n = 13)

The surveyed parameter	Characeristic of group of examined donors	x ± SE	Median	25%–75%	Min–Max
Content of lymphocytes, out of their total amount, with plasmatic FN on the surface, %	control group of clinically healthy donors	53.4 ± 1.4	54.2	49.8–57.3	41.9–59.4
	group of patients with steatohepatitis	59.3 ± 2.2	58.9	52.2–65.9	46.2–70.9
Content of lymphocytes, out of their total amount, with cellular FN on the surface, %	control group of clinically healthy donors	88.6 ± 1.4	88.0	85.0–90.0	81.0–100.0
	group of patients with steatohepatitis	36.9 ± 1.3***	36.0	35.0–41.0	28.0–45.0
Contents of lymphocytes, out of their total content, with α_5-integrin subunit, %	control group of clinically healthy donors	97.6 ± 0.4	98.0	96.6–98.4	95.0–100.0
	group of patients with steatohepatitis	98.7 ± 0.2	98.7	98.2–99.1	97.0–100.0
Intensity of exposure of plasmatic FN on the surface of lymphocytes, mV	control group of clinically healthy donors	54.3 ± 1.3	55.3	52.3–57.2	41.9–59.3
	group of patients with steatohepatitis	53.2 ± 0.7	52.4	51.5–54.6	50.2–59.3
Intensity of exposure of cellular FN on the surface of lymphocytes, mV	control group of clinically healthy donors	36.1 ± 0.6	36.5	34.5–37.5	32.0–39.5
	group of patients with steatohepatitis	119.8 ± 1.2***	120.0	116.0–122.0	112.0–128.0
Intensity of exposure of α_5-integrin subunit on the surface of lymphocytes, mV	control group of clinically healthy donors	87.2 ± 0.9	87.0	85.0–90.0	82.0–93.0
	group of patients with steatohepatitis	428.0 ± 1.8***	429.0	425.0–433.0	413.0–437.0

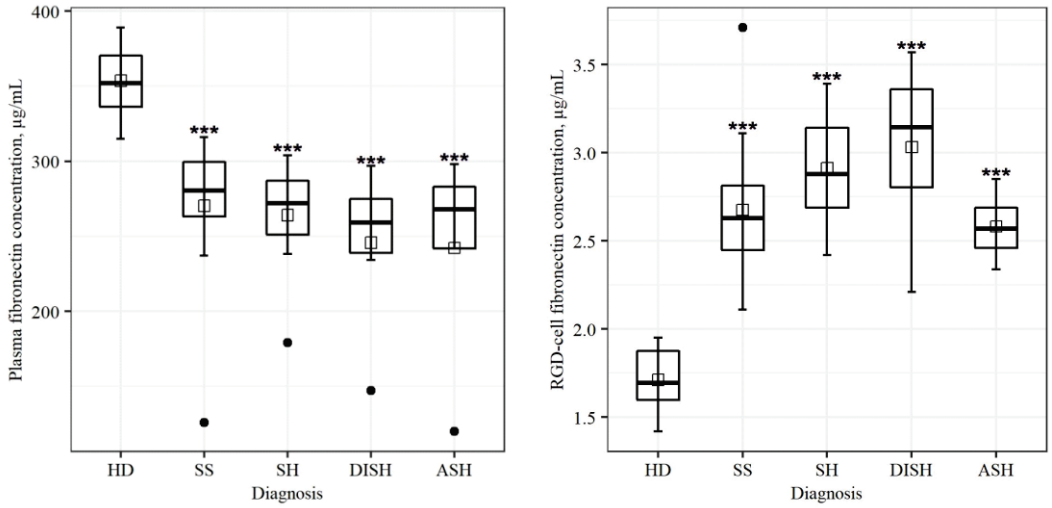
Note: *** – P < 0.001 compared with the control group (PHD) according to Tukey test with Bonferroni correction.

In the groups of ASH and SS we found patients with significantly decreased parameters of pFN concentration measuring respectively 120 and 126 μg/mL, which corresponds to an almost two-fold decrease in the content compared with the HD group. All the samples from the groups of patients with ASH and DISH displayed pFN content lower than the norm, while in the NAFLD group 25% of the samples of plasma samples from the patients had the parameters which corresponded to the normal content of pFN. For the cellular form of fibronectin we determined concentration in the blood plasma for the group of clinically healthy donors equaling 1.71 ± 0.05 μg/mL. At the same time, we recorded increase in the concentration of cellular fibronectin in the plasma in the presence of CDLD compared with the norm measuring on average 63.8% (Fig. 2b). The greatest increase in the cFN content at the level of 77.2% was observed in patients suffering from drug-induced hepatitis, whereas in the groups of SS, SH and ASH the content of cellular fibronectin increased respectively by 56.7%, 70.2% and 50.9%. Maximum increase in the concentration of plasmatic cFN compared with the control values was determined in patient of NAFLD group, equaling 217.2%. Analysis of the parameters of concentrations of pFN and cFN between the groups of patients suffering from chronic diffuse liver diseases revealed no statistically significant differences (P > 0.05). Statistical characteristics of diagnostic possibility of the content of plasmatic and cellular FN in plasma, determined using the analysis of ROC-curves, indicate the excellent informativeness of these parameters. Test of concentration of cFN has maximum diagnostic efficiency, Se = 1.0, Sp = 1, AUC = 1. Cutoff point corresponds to the content of protein in plasma at the level of 2.11 μg/mL, excess of which may predict pathology. For plasmatic FN, we determined critical concentration measuring 315 μg/mL (Youden index equaling 0.943), decrease in the concentration below this level may indicate possibility of pathology. Se = 1.0, Sp = 0.944, AUC = 0.944 (P < 0.001).



a *b*

Fig. 1. Intensity of fluorescence (mV) of antibodies to cellular fibronectin (*a*) and integrin subunit (*b*) on the surface of lymphocytes in clinically healthy donor (black line) and patient with chronic diffuse liver disease (red line) according to the data of flow cytometry on Beckman Coulter EPICS; density of exposure was calculated in FCS Express 3 software



a *b*

Fig. 2. Concentrations of plasmatic (*a*) and cellular (*b*) fibronectin in blood plasma of the groups of clinically healthy donors (HD) and patients with chronic diffuse liver diseases: SS – patients with steatosis, SH – patients with steatohepatitis, DISH – patients with drug-induced hepatitis, ASH – patients with alcoholic hepatitis; *** – P < 0.001 compared with the control group (HD) according to Tukey test with Bonferroni correction

Analysis of ROC-curves of the tests of immune cells displayed the greatest efficiency of the following tests: content of lymphocytes with surface-associated cellular FN, intensity of exposure of cellular FN on the surface of lymphocytes and intensity of exposure of α_5 on the surface of lymphocytes: Se = 1.0, Sp = 1, AUC = 1. Cutoff points equal respectively: 88.0%, 112 mV and 413 mV. Test for the content of lymphocytes with associated plasmatic isoform of FN on the surface indicated the following data: Se = 0.615, Sp = 0.46, AUC = 0.722 (P = 0.033), good diagnostic accuracy, cutoff point of 57.8%. Test of the content of lymphocytes, out of the total amount, with α_5-subunit on the surface indicated reliability at the level: Se = 0.769, Sp = 0.692, AUC = 0.742 (P = 0.016), good diagnostic accuracy, cutoff point equaling 98.2%. Average diagnostic reliability was exhibited by test of intensity of exposure of plasmatic FN on the surface of lymphocytes: Se = 0.769, Sp = 0.615, AUC = 0.672 (P = 0.136), cutoff point of 53.2 mV (Fig. 3).

The greatest corelational dependence was detected between the tests of intensity of exposure of plasmatic form of fibronectin on the surface of lymphocytes and the content of lymphocytes which express α_5-subunit: the relationship of correlation was reverse, Spearman coefficient was very close to the lower value of the range with strong correlational relationship ($\rho = -0.6804$; P = 0.011). A positive correlational relationship of average strength was determined between the test of the level of concentration of cellular FN in plasma and the intensity of exposure of cellular FN by lymphocytes ($\rho = 0.5517$; P = 0.049). We should also note the direct correlational average-strength dependence between the intensity of exposure of cellular FN on lymphocytes and the intensity of exposure of integrin subunit by lymphocytes and concentration of cFN in plasma, though statistical levels of significance in those tests exceed the threshold value (P > 0.05).

Analysis of possibility of predicting presence of CDLD using the model of logistic regression revealed that the highest effectiveness was displayed by the following tests: intensity of exposure of α_5-subunit on the surface of lymphocytes, intensity of pFN exposure on surface of lymphocytes, intensity of cFN exposure on the surface of lymphocytes, concentration of plasmatic FN in blood plasma, concentration of cellular FN in blood plasma. Error of prediction using these parameters equals zero. Other survey tests had efficiency of prediction of around 60–70%, namely: error of prediction for the content of lymphocytes with activated α_5-subunit on the surface equaled 27.1%, for the content of lymphocytes with membrane-associated pFN on the surface – 38.5%, for pFN exposure on lymphocytes – 30.8%. Form of the graphic curves allows us to evaluate the predictive ability of the tests (Fig. 4).

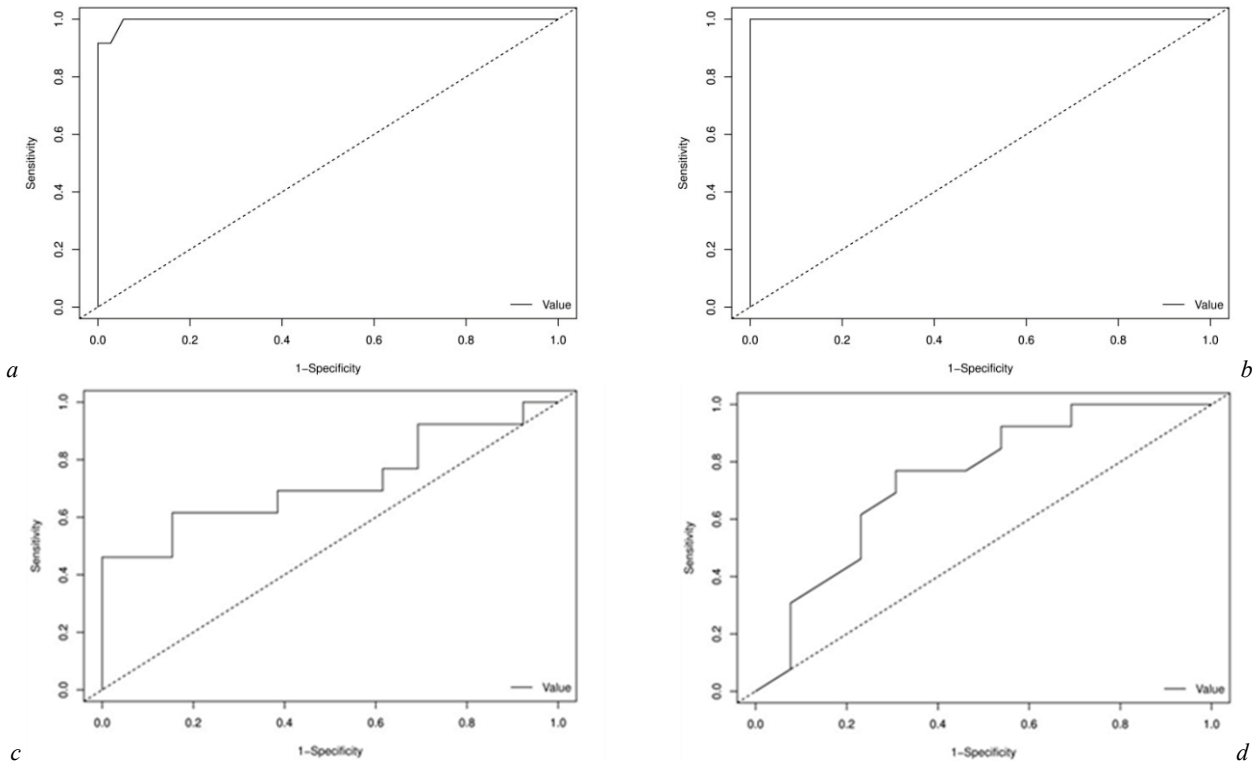


Fig. 3. ROC-curves of use of informativeness of parameters of the levels of pFN concentration in blood plasma (*a*), level of cFN concentration in plasma (*b*), share of lymphocytes, out of their total amount, with membrane-associated plasmatic FN on the surface (*c*) and content of lymphocytes with membrane-associted α_5-integrin subunit on the surface (*d*)

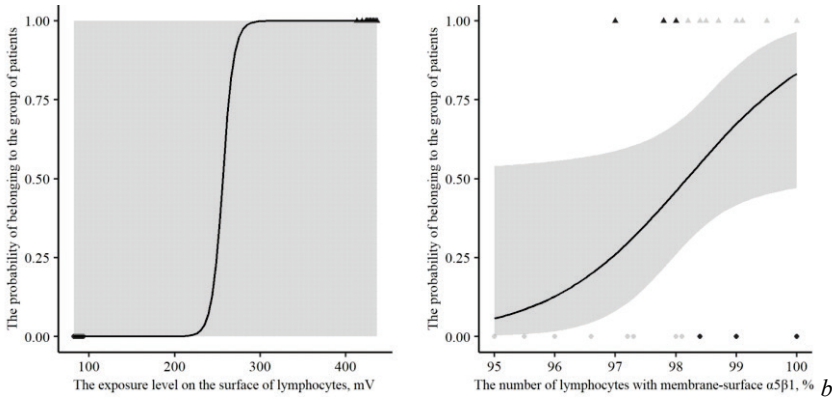


Fig. 4. Logistic curves of prediction of presence of CDLD using model of logistic regression for the tests: intensity of exposure of α5-subunit on the surface of lymphocytes; wrongly classified objects on 4b graph are indicated with dark colour

In the case of zero error, the graph has an ideal form of symmetric curve with saturation (Fig. 4a). Such a view indicates possibility of clear division of norm-pathology classes. The logistical curve depicted in Figure 4 corresponds to five surveyed tests: exposure of α_5-subunit on the surface of lymphocytes, exposure of plasmatic fibronectin on the surface of lymphocytes, exposure of cellular fibronectin on the surface of lymphocytes, concentration of plasmatic fibronectin in blood plasma, concentration of cellular fibronectin in blood plasma. If the prediction ability decreases, logistic curve becomes graphically close to a straight line (Fig 4b). Graphs of logistic curves for the tests: content of lymphocytes with surface-associated integrin α_5-subunit, content of lymphocytes with plasmatic fibronectin on the surface and content of lymphocytes with plasmatic fibronectin on the surface are close to a straight line, indicating the low prediction ability of the surveyed parameters for predicting presence of chronic diffuse liver diseases.

Discussion

Chronic diffuse liver diseases as a result of long activation of the healing reaction are characterized by several important traits: continuous chronic damage of hepatocytes; accumulation of complex inflammation infiltrate in the liver tissues, blood and lymph, activation of different types of ECM-producing cells with proliferative, synthetic and contractile abilities (Svegliati-Baroni et al., 2008). A vitally important role in the process of wound healing is played by fibronectin which is a profibrinogenic protein which induces the expression of collagen genes and precedes the deposition of other ECM components in the process of liver fibrognesis (Rodríguez-Juan et al., 2009). Plasmatic FN circulates in the blood in inactive form and is stored in α-granules of thrombocytes until being activated by reaction to a wound. The main sources of plasmatic fibronectin are liver hepatocytes, from where it is secreted into the blood flow for distribution across the whole organism. Cellular fibronectin is produced both locally in the tissues, and is synthesized by various cells, assembles in complex fibrillary matrix on the surface of cells and regulates deposition of other ECM proteins, and also migration, adhesion, differentiation of cells, etc. (To & Midwood, 2011). In the normal physiological conditions, there is a balance between both types of fibronectin in the blood flow and intact tissue, whereas in certain conditions of inflammation or damage, the contents of both pFN and cFN may significantly change.

The data we obtained indicate benefits from the fact that chronic diffuse liver diseases of non-virulent etiology in most cases are accompanied by decrease in the content of plasmatic form of FN in blood flow. Decrease in concentration of the protein was determined for almost all patients suffering from steatosis which usually is asymptomatic stage of the disease. As the disease develops, the number of patients with reduced FN concentration increased. Therefore, in the group with steatosis, for 32% of the patients the content of plasmatic FN was at the level of the normal values (300–400 μg/mL), while in the group with steatohepatitis this indicator decreased to 7.6%. In practically all the surveyed groups, we found patients with critical decrease in the protein content, depicted as burst (Fig. 3). Our conclusions coincide with the data of other researchers. Therefore, fall in the level of plasmatic FN was recorded during the survey of patients with liver cirrhosis, chronic active hepatitis, chronic persisting hepatitis (Levitan & Astakhin, 2012). These authors also report that in patients with CDLD with liver failure, decrease in concentration of plasmatic FN below 100 μg/mL was predictively extremely unfavourable, requiring immediate therapeutic measures. Decrease in pFN concentration in patients with CDLD may be related both to increase in consumption from the blood flow, as well as possible decrease in the protein-synthesizing function of the liver in these patients (Levitan & Astakhin, 2012). At the same time, all the groups of patients were observed to have significant increase in the content of cellular FN. As known, in normal conditions cFN is a harmless and secondary component of ECM. However, its concentration significantly increases in the conditions of embryonic development, during the events that include regeneration or restoration and in the conditions of various chronic diseases characterized by significant damage to the tissues. During the restoration of integrity of the tissues, production of cFN usually decreases to the homeostatic levels. However, in the conditions of impairment of the functions of the tissues and regulatory mechanisms which limit the activity of cFN, the expression of protein becomes constant, which in turn may cause more damage to the tissues than restoration (Aziz-Seible & Casey, 2011). Increased production of cFN by hepatic perisinusoidal cells and malfunctioning of its removal by hepatocyte-specific ASGP-R way was reported for the conditions of alcoholic hepatitis. At the same time, on the one hand, activation of cFN causes intense activity necessary for restoration of damaged tissue. On the other hand, in the conditions of malfunctioning of regulatory mechanisms which limit cFN activity, the condition of unstoppable "wound healing" may develop, leading to even more damage (Aziz-Seible & Casey, 2011). In our study we determined increase in concentration of cellular FN already at the stage of steatosis, and when inflammatory damage increased the cFN concentration in blood increased. If during the steatosis stage, 21.2% of patients were observed to have cFN concentration in plasma corresponding to the normal concentration (1.5–2.0 μg/mL), patients of other groups had increased content of protein.

The studies on the level of distribution of both forms of fibronectin and its integrin receptor on the surface of cells of immune system were performed using fraction of lymphocytes. This fraction of lymphocytes displayed excellent diagnostic informativeness in studying samples of plasma of oncopatients in our previous experiments (Netronina et al., 2018). The level of exposure of cellular fibronectin and α_5-integrin subunit in the surface of lymphocytes was found to significantly increase. According to the data of polymerase chain reaction the level of ITGA5 expression reliably did not differ from the control values, whereas during onco-proliferative processes our studies revealed changes in FN1 expression (Kulinich et al., 2010). According to the results of the surveys, the number of lumphocytes with membrane-associated cellular FN significantly increased over inflammatory processes, namely chronic diffuse liver diseases of non-virulent etiology.

As known, integrins which express in circulating lymphocytes are inactive and do not provide adhesion, In the conditions of inflammatory processes, the cells become subjected to the influences of various irritators, including chemokines which quickly activate the function of integrins through modulation of LFAq, which is an antigene related to the function of lymphocytes (Kinashi, 2005). Activation of $\alpha_5\beta_1$ leads to change in conformation of integrin, emergence of high affinity to its classical ligand of fibronectin way of recognizing Arg-Gly-Asp motif and underlies the creation of a strong stable relationship and avidity of the complex (Chan et al., 2003). In these adhesive complexes, cytoplasmatic tails of $\alpha_5\beta_1$ are related with active cytoskeleton and internal signal molecules, which is the key for the assemblage of the matrix. Interaction of integrins with cytoskeleton allows the receptors to move through active filaments, unwinding the bound molecules of fibronectin and thus stimulating fibrilogenesis. In turn, the structure of matrix is essential for further cellular events (Machado-Pineda, 2018).

Exchange of information between inside and out occurs through activation of specific signaling molecules such as FAK and Rho (Schwarzbauer & DeSimone, 2011). Functional responces of leukocytes which occur as a result of tranduction of signals outside-in include migration, proliferation, secretion of cytokins, degranulation (Abram & Lowell, 2009). Increase in the level of exposure of $\alpha_5\beta_1$ on one hand and its activation on the other hand require increased demand for fibronectin, which may be one of the reasons for decrease in plasmatic Fn in plasma, because particularly this soluble form of glycoprotein of plasma is first to contact the activated $\alpha_5\beta_1$ lymphocytes with following initiation of unfolding of globular structure and open domains for support of polymerization (Zhou et al., 2008). In turn, pFN monomers initiate $\alpha_5\beta_1$-dependent signaling cascades, for expression of cellular isoforms of EDA, EDB. Therefore, the study on expression of FN forms by endothelial cells revealed that fibrillogenesis of FN on cells first of all requires obligatory endogenic production of matrix protein, and secondly that only polymerization of cellular forms of FN provides enough adhesive ligands for productive interaction with cellular receptors and components of the matrix, increases bioavailability of antiogenic factors, including VEGF and FGF-2 (Cseh et al., 2010). The survey of lymphocytes in our laboratory revealed that in conditions of chronic diffuse liver diseases, the exposure of cellular FN increases by 3.3 times compared with the control group, and α_5-integrin subunit increases by 4.9 times.

The advantages of non-invasive methods of monitoring of chronic diffuse liver diseases include absence of complications and contraindications, safety, possibility of use for assessment of dynamics. Currently, there is known a whole panel of serologic biomarkers of CDLD, particularly: alamine amino transferase (ALT), aspartate aminotransferase (AST), hyaluronic acid, I and III types procollagen peptides, IV type collagen, growth factor β (TGF-β), matrix metaloproteinases MMP-2 and MMP-9, inhibitor of metalloproteinase-1 (TIMP-1), etc. These markers are most relevant and are traditionally applied to diagnose chronic diffuse liver diseases (Didenko et al., 2014), but the search for new indicators with sufficient level of sensitivity and specificity continues. Such a search for plasmatic and clinical markers based on the data of hematological analysis has been made by the staff of our laboratory for many years. We surveyed the levels of mRNA fibronectin expression and its exposure on lymphocytes, monocytes and granulocytes of blood plasma in conditions of cirrhoses and fibrosis; activity of cysteine cathepsins in blood plasma and inhibitors of proteolysis of α1-antitrypsin and α2-macroglobuline (Dolgikh et al., 2020). According to the results of the presented survey, we found statistically significant changes in the concentrations of plasmatic and cellular forms of fibronectin in blood plasma of patients with CDLD of non-virulent etiology, level of exposure of cFN, pFN and α_5-subunit on the surface of cells of immune system of lymphocytes. These indicators can be proposed for further surveys with the purpose of creating new biomarkers based on them.

Conclusions

Use of biochemical and molecular-biological methods such as flow cytofluorimetry, polymerase chain reaction, ELISA and Western-blotting allowed us to study free and cell-bound forms of fibronectin, its integrin receptor in the group of patients with chronic diffuse liver diseases of non-virulent etiology. Such complex approach allowed us not only to compare and evaluate these parameters, but also to differentiate the most important of them from the diagnostic perspective using modern methods of statistical analysis. According to ROC-analysis, the levels of plasmatic and cellular fibronectins have diagnostic possibility and excellent predictive informativeness. At the same time, the patients with CDLD had opposite changes in their contents – the level of pFN decreased, while the level of cellular FN, by contrast, increased. The obtained results provide a basis for further studies, particularly the search for coefficients of relationships between these parameters using a broader variety of selections of patients. Using the model of logistic regression the greatest efficiency in predicting CDLD wasseen in the parameters obtained using flow cytofluorimetry: particularly increase in the levels of exposure of α_5-integrin subunit and cellular fibronectin on blood lymphocytes.

The results of the surveys allows us to propose the abovementioned parameters as additional ones for diagnosing chronic diffuse liver diseases, especially their asymptomatic stages using only the patient's blood, thus avoiding paracentesis, which has a number of side-effects.

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