Eprosartane, in the presence of contraindications for angiotensin II - converting enzyme inhibitor Ramipril, is absolutely opportune in the presence of hypertensive microalbuminuria.

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Cathepsin D Activity in Experimental Liver Chirrosis and After the Administration of Copper Coordination Compounds and Bacterian Remedy BioR

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Abstract

This paper investigates the influence of the copper coordination compounds CMT-28, CMT-67 and of the bacterial remedy BioR on the cathepsin D activity in liver in experimental cirrhosis. The activity of cathepsin D was also detected electron-histochemicaly in the liver during the regression of experimental hepatic cirrhosis. The result suggests that the coordinative compound CMT-67 used in combination with the bacterial remedy BioR has a pronounced stimulating effect on the enzymatic hydrolysis of the extracellular matrix under the action of cathepsin D and contributes to a more efficient breakdown of the excessive fibrous tissue in liver. It was determined that the active cathepsin D is localized intracellularly in the lysosomes of hepatocytes, macrophages, fibroblasts and endothelial cells, as well as extracellularly on the collagen fibrils near the parenchymal and mezenchymal cells. In addition to its participation in the intracellular proteolysis, cathepsin D is secreted by the hepatocytes and connective tissue cells to the extra-cellular space and participates in the extracellular breakdown of the fibrous tissue.

Key words: cathepsin D, liver cirrhosis, coordinative compounds of cuprum, bacterial remedy BioR.

Активность катепсина D при экспериментальном циррозе печени и при введении координационных соединений меди и препарата бактериального происхождения BioR

Было изучено влияние координационных соединений меди CMT-28, CMT-67 и препарата бактериального происхождения BioR на активность катепсина D в печени при экспериментальном циррозе. Активность катепсина D была также выявлена электронно-гистохимически в печени при регрессии экспериментального цирроза. Результаты свидетельствуют о том, что координационное соединение меди CMT-67, введенное в комбинации с препаратом бактериального происхождения BioR, имеет выраженное стимулирующее влияние на ферментативный гидролиз внеклеточного матрикса под влиянием катепсина D и способствует более эффективному распаду фиброзной ткани в печени.

Было выявлено, что активный катепсин D локализуется внутриклеточно в лизосомах гепатоцитов, макрофагов и эндотелиальных клеток, а также внеклеточно – на коллагеновых волокнах вблизи паренхимальных и мезенхимальных клеток. Таким образом, помимо участия во внутриклеточном протеолизе, катепсин D секретируется гепатоцитами и клетками соединительной ткани во внеклеточное пространство и участвует во внеклеточном распаде фиброзной ткани.

Ключевые слова: катепсин D, цирроз печени, координационные соединения меди, препарат бактериального происхождения BioR.

Introduction

Hepatitis and cirrhosis are regional pathologies of Moldova. Morbidity and mortality caused by these diseases are constantly growing. Liver cirrhosis is a chronic diffuse liver disease with diverse etiology, characterized by inflammatory, degenerative, necrotic and concomitant regenerative processes, accompanied by progressive disturbances of the organ structure and function [1]. The etiologic factor causes dystrophy and necrosis of the liver cells, infiltration of the portal ducts, cholestasis and connective tissue development. Hepatocytes necrosis is followed by the regeneration of some of the remaining liver cells that leads to the formation of the pseudolobules, which substantially disrupt the normal structure of the organ, create circulatory problems that results in severe deficiencies in oxygen and nutrients supply to the hepatocytes and their continuous damage [2]. Fibrosis – the deposition of fibrous connective tissue in excess, which replaces necrotizing parenchyma elements, is the consequence of most liver diseases arising from chronic aggression carried out by various agents (viral, toxic, immunologic, metabolic) [3]. Fibrosis is characterized by a 3-6 fold increase in the amount of all extracellular matrix components, some of which will increase disproportionately and induce subtle changes of the microstructure at the molecular level [4, 5]. It is believed that the essential cause of the excessive accumulation of connective tissue in the liver is the impaired balance between synthesis and degradation of liver extracellular matrix components, in particular, collagen, occurring as a result of parenchyma damage, blood circulation deficiencies (including, resulting hypoxia) caused by the products of the unpaired metabolism. Ultimately the self-regulation of the connective tissue is affected and the biosynthesis of the compounds of the extracellular matrix begins to outweigh their catabolism, which provides further progression of fibrosis [6]. It is well known that fibrogenesis insured by the mezenchimal component is balanced by fibrolysis that is controlled by the parenchyma cells. Thus the continuing damage of the parenchyma can unbalance the fibrogenesis and fibrolysis and evolve to fibrosis [7].

When the cell degeneration starts, the reparative processes also begin. Destructive and reparative processes run simultaneously for a long time, thus the necrotized liver tissue is replaced by functionally active one. Mechanisms, which provide structural and functional restoration of the liver in such conditions, particular reparative metabolic processes in the damaged liver tissue are still unknown.

Lysosomal apparatus of the cell with its powerful complex of hydrolases, in particular, cathepsins B, D, G, L, H, possesses a particularly important role in adaptive changes of the disturbed metabolic processes and structure of organs and systems due to the action of exogenous chemicals [8, 9]. Protective function of lysosomes is manifested by their involvement in the intracellular digestion of phagocytized macromolecular and supramolecular structures that a sensed as foreign compounds as well as degraded intracellular structures. Frequently extreme factors increase the activity of intracellular proteolytic enzymes, which lead to the formation of biologically active compounds that influence the biosynthesis of proteins and nucleic acids [10].

Cathepsin D, considered to be a marker of the lysosomal enzymes as acidic phosphatase, is one of the most important lysosomal aspartic proteinase and is capable to degrade the main components of the intercellular matrix: collagen, proteoglycans and glycoproteins (fibronectin). Cathepsin D attacks the non-helical terminal regions of the collagen molecules or the α-helical chains, digesting the solubile colagen and solubilizing about 10% of the unsoluble colagen at pH 3.3-4.0.

Cathepsin D is involved in the degradation of proteoglycans at pH 5.0. The efficacy of the proteolysis, producer by cathepsin D is highest at acidic pH values (2.8 to 5.0), although some authors record *in vitro* high enzymatic activity and a pH of 7.2. This allows supposing that cathepsin D participates not only in the intracellular degradation of the proteins, but also in their extracellular catabolism, but the later is not proven.

Since the cellular lysosomal system is an important part in the body's enzymatic protection against the aggression of foreign substances, the study of the mechanisms of action of coordination compounds of transition metals and cyanobacterial remedies on lysosomal hydrolyses in the regression of experimental liver cirrhosis, is of particular interest especially on purpose to use them as versatile medical preparations for the morpho-functional recovery of organs affected by fibrosis and sclerosis.

The objectives of the study were:

1) The assessment of the changes in cathepsin D activity in experimental liver cirrhosis (CH) and after the administration of copper coordination compounds (CC) and their combinations with BioR.

2) Electron-histochemical detection of cathepsin D activity in the liver in the process of regression of experimental liver cirrhosis.

Material and methods

The biological activity of copper coordination compounds CMT-28 and CMT-67 and of their combinations with cyanobacterial remedy BioR were evaluated in the experiment on a group of animals consisting of 80 male white rats weighing 200-220g, divided into 8 groups of 10 animals each. The first group-control, consisted of 10 animals, maintained on a normal vivarium diet and treated with normal saline that was injected intramuscularly daily. Group No. 2-8 consisted

of experimental animals that were injected intramuscularly 50% sol. of carbon tetrachloride (CCl_4), 1 mg/kg twice a week, over 60 days to induce experimental liver cirrhosis. Carbon tetrachloride (CCl_4) enters the body and reacts with amines and proteins resulting in the formation of free radicals. Lipid peroxidation by free radicals disturbs the function of the cell membranes, including the lysosomal membrane. This will increase the permeability that is considered a universal mechanism of cellular damage at membrane level [11, 12].

Animals in group 3 were treated with CMT-28, while those in group 4 - the CMT-67. Animals from group No. 5 were subjected to treatment with CMT-28 in combination with BioR, and group 6 - CMT-67 in combination with BioR. All those preparations were administered intramuscularly for 14 and 28 days, the daily dose being of 1.0 g/kg body weight.

Animals in groups 1-6 were sacrificed under light narcosis with sulfuric ether 24 hours after the last injection. Animals in groups 7 and 8 were sacrificed 7 and 14 days, respectively, after the suspension injections of CCl_4. Biological material - the liver, was collected, washed with 0.85% sol. of NaCl and dried with filter paper. Further liver homogenate was prepared in 0.25 M sucrose buffer, containing 1 mM EDTA, pH 7.4, so the final dilution of homogenate to be 1:10.

Cathepsin D activity was determined in liver homogenate according to the procedure described by Barrett A., 1977 [13], modified by Gudumac V. [14]. The principle of this method is based on the enzyme's ability to make an intense hydrolysis with the formation of hemoglobin macromolecule acid-soluble derivatives, which can be estimated spectrophotometrically. Statistical evaluation of biochemical indices was made by parametric t-Student criterion with reliability less than 0.05 ($p < 0.05$).

For electron-histochemical determination of cathepsin D activity in animal liver at ultra structural level the liver of the rats from groups 7 and 8 was processed in accordance with the procedure described by Smith and Van Frank [15]. Tissue samples with dimensions 0.5×0.5×1.0 mm were fixed for 3 hours in 0.05 M cacodilat buffer, pH 7.2 with 1.5% glutaraldehyde. Then during three days the samples were washed in 0.05 M cacodilat buffer, pH 7.2, containing 7% sucrose. The substrate for incubation was BZ-Arg-Gly-Phe-Phe-Pro-4MβNA (Bachem). Incubation lasted 30 min at 37˚C in the medium, which contained 24 mg substrate dissolved in 1 ml dimethylformamide and 25 ml glycine-HCl buffer, pH 3.1. The reaction is stopped by the addition to the reaction medium of 10% KOH with subsequent washing with HEPES buffer, pH 7.0 and was followed by the incubation in pH 5.4 cacodilate buffer with dipeptidil-aminopeptidase II and para-roseaniline at 37˚C for 15 min. After the incubation the material was fixed with 1.5% sol. osmium tetraoxide in cacodilat in buffer (pH 7.2) for 90 min. In the usual manner for electron microscopy examination, the exploratory material was dehydrated in ethyl alcohol solutions with increasing concentrations (50%, 70%, 96%, and 100%) and then incubated for 20 min in absolute acetone. Samples were included in the eponymous and left in the thermostat at 60˚C for 24 hours. As reference, material was used the tissue incubated without substrate.

Results and discussions

The results of the research, illustrated in figure 1, have shown that in animals with experimental liver cirrhosis induced by CCl_4, activity of cathepsin D increase statistically significant by 27% compared to the control group. Knowing the properties of this proteolytic enzyme and its ability to initiate protein degradation, in particular collagen, we can assume that the increased activity of cathepsin D at the stage of maximal cirrhosis development is a manifestation of the fact that in the liver, parallel with fibrogenesis process runs fibrolysis for the degradation of the excessive connective tissue.

Administration of the bioremedy BioR in dose of 1 mg/kg did not affect the enzyme activity, maintaining it practical at the control levels. At the same time, administration of BioR in the dose 2 mg/kg maintains the enzyme activity at levels

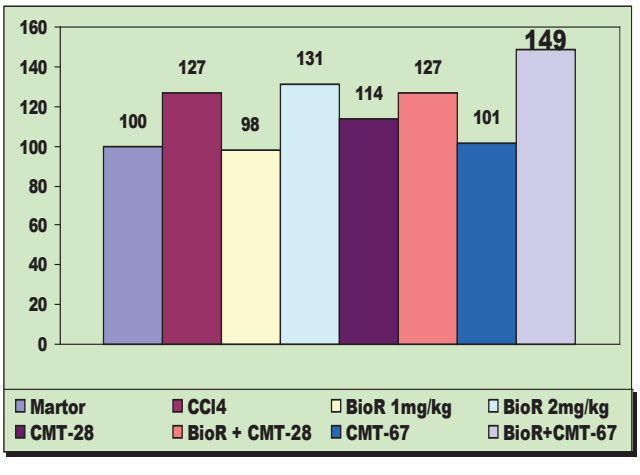

Fig. 1. Changes in cathepsin D activity (%) in liver cirrhosis and administration of CC and their combinations BioR.

similar to those assessed in the group of animals with LC and is 31% higher then the control levels. After the treatment with copper coordination compound CMT-28 the activity of cathepsin D showed a tendency to decrease by 10% in animals intoxicated with CCl_4. At the same time, the combined use of CMT-28 and BioR does not change the functional level of the enzyme in the liver of the animals with cirrhosis, which was by 27% higher comparative with the original level. The copper coordination compound CMT-67 reduces the degree of cathepsin D activity induction, triggered by cirrhosis, maintaining it at normal values. At the same time combined medication with CMT-67 and BioR increases statistically significant the enzyme activity by 49% ($p < 0.05$) compared with healthy animals and by 17% compared with rats with cirrhosis.

The enhancement of the activity of cathepsin D under the combined influence of CMT-67 and BioR can be considered as a compensatory adaptation reaction of the body, which tends to amplify the biodegradation of defective molecules, resulting from harmful action of CCl_4 on liver tissue. The combined administration of copper coordination compound CMT-

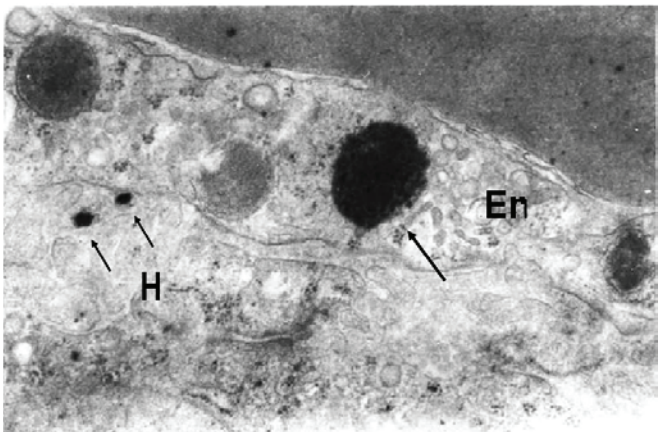

Fig. 2. 14-th day of experimental cirrhosis regression. A pronounced reaction on cathepsin D (arrows) in the lysosome of endothelial cell (En), and extracellular on hepatocytes microvilli (H). x 20000.

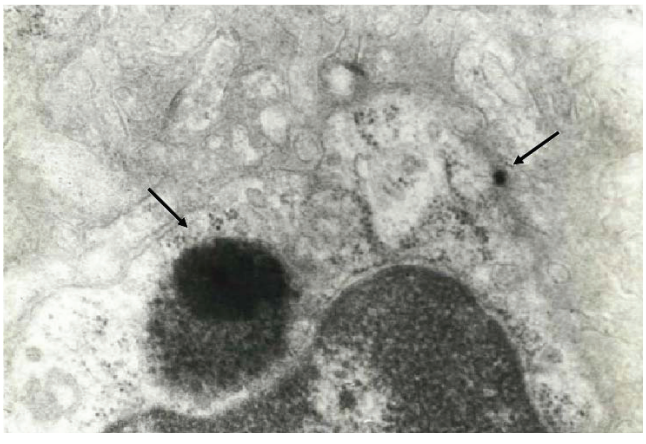

Fig. 3. 14-th day of experimental cirrhosis regression. Reaction on cathepsin D (arrows) in the lysosome of Kupffer cell, and extracellular on the cytolemma. x 20000.

67 with cyanobacterial remedy BioR exercise pronounced stimulatory effect on the process of enzymatic hydrolysis of liver extracellular matrix compounds, contributing to a more efficient degradation of fibrous tissue, which is demonstrated by inducing the expression of cathepsin D. The results of the study showed that the investigated remedies have selective action on cathepsin D activity.

Electron-histochemical detection of cathepsin D activity in the liver in the process of experimental liver cirrhosis regression. In order to elucidate the participation of cathepsin D in the resorption of the connective tissue in the liver, we performed electron-histochemical detection of cathepsin D. We studied the distribution of the enzyme activity in the liver at the ultrastructural level within two weeks after cessation of intoxication.

The cathepsin D reaction product was detected in the lysosomes of the hepatocytes, macrophages and fibroblasts 7 and 14 day after cessation of CCl_4 administration by electron-histochemical investigation. A pronounced reaction was detected in the lysosomes of endothelial cells, too (fig. 2). It was noted a marked heterogeneity in the distribution of reaction product within different cell types and between lysosomes belonging to the same cell. A maximal expressed activity was seen in macrophages (fig. 3) and fibroblasts in all periods of investigation. The activity of cathepsin D was often detected in the myelin-type structures and in the autophagy vacuoles of the hepatocytes after 7 days of regression of cirrhosis.

A major result of our study was the electron-histochemical detection of extracellular activity of cathepsin D at both studied stages of cirrhosis regression. The reaction product is preferentially located on the collagen fibrils near the hepatocytes and cellular elements of connective tissue and, also, on the hepatocytes microvilli and on the external surface of the Kupffer cells cytolemma (fig. 2, 3). Therefore, both parenchymal cells and connective tissue cellular elements are sources of extracellular cathepsin D.

The electron-histochemical investigations of samples of liver tissue in the process of regression of cirrhosis have revealed the intracellular localization of cathepsin D - in the lysosomes of the hepatocytes, macrophages, fibroblasts and endothelial cells, and the extracellular localization - on the collagen fibrils near the parenchymal and mesenchyme cells. The heterogeneity of distribution and intensity of the reaction product, observed in our study reveals different functional status of the lysosomal system, belonging to different cell types.

Extracellular cathepsin D activity detected in the liver damaged by cirrhosis reveals that besides being involved in intracellular proteolysis, cathepsin D is secreted by hepatocytes and cellular elements of connective tissue in the intercellular space and participates in the catabolism of hepatic extracellular matrix and extracellular resorption of fibrous tissue. The important role of cathepsin D was established in liver cell division process during liver regeneration [10].

Therefore, increased activity of the studied proteinase during the regression of the liver pathology could be required for both degradation of connective tissue formed in excess and/or damaged cell structures, and to provide processes of cell division.

Conclusions

1. Administration of the combination of copper coordination compound CMT-67 with cyanobacterial remedy BioR exhibited a pronounced stimulatory effect on the enzymatic hydrolysis processes of liver extracellular matrix under the action of cathepsin D, contributing to the more efficient degradation of the excessive fibrous tissue.

2. Active cathepsin D is localized intracellular in the lysosomes of the hepatocytes, macrophages, fibroblasts and endothelial cells and extracellular – on the collagen fibrils near the parenchymal and mesenchyme cells.

3. In addition to its involvement in the intracellular proteolysis, cathepsin D is secreted by the hepatocytes and connective tissue cellular elements into the intercellular space and participates in the extracellular resorption of the fibrous tissue.

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Macrophage Density Correlates with Severity of Uterine Cervix Neoplasia

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Abstract

Despite all recent efforts, cancer of the uterine cervix still remains one of the most frequent malignancies among women. Lymphatic vessels represent the primary route of tumor cells dissemination in cervical cancer. It has been demonstrated that cervical neoplasia actively participates in the recruitment of new blood and lymphatic vessels. Macrophages are extremely versatile cells which have a significant contribution to tumor progression. **The aim:** 1) To establish the correlation between tumor-associated macrophages (TAM) and the grade of the uterine cervix neoplasia; 2) To evaluate the distribution of TAM within both intratumoral and peritumoral areas. **Material and Methods:** Ninety-six cases were studied. The specimens were fixed in buffered formalin and paraffin embedded. Step sections, 5μm thick, were performed for each case. Initial sections were stained with haematoxylin-eosin, for the pathological diagnosis and grading of the tumor. Lesions were classified as follows: squamous cell metaplasia (n = 12), CIN I (n = 8), CIN II (n = 6), CIN III (n = 24), microinvasive carcinoma (n = 16) and invasive squamous cell carcinoma (n = 26). Additional sections for each case were stained for CD68 antibody, in order to highlight the macrophages. Quantification of macrophage population has been made based on hot-spot technique. The arithmetic media of 3 (× 200) fields represented the final result. **Results:** We found a statistical correlation between peritumoral macrophages (PTM) and intratumoral macrophages in all stages of cervical neoplasia, macrophage density and tumor stage ($p = 0.01$). In 16 cases we found vascular invasion. Almost in all these cases (87.5%) intravascular tumor emboli were embedded with CD68+ cells. **Conclusions:** based on these findings, we consider that macrophages are key regulators of cervical cancer progression. TAM targeted management could be an essential therapeutic strategy, not only in order to suppress the progression of cervical neoplasia, but also to inhibit macrophage-mediated vascular invasion.

Key words: uterine cervix cancer, macrophages, cellular density, tumor progression, CD68.

Корреляция плотности макрофагов с тяжестью неоплазии шейки матки

Рак шейки матки остается одной из самых часто встречающихся злокачественных заболеваний женского населения. Лимфатические сосуды являются первичным путем метастазирования при данном заболевании. Было доказано, что клетки цервикальной неоплазии активно участвуют в образовании новых лимфатических сосудов. Макрофаги – многофункциональные клетки, оказывающие большое влияние