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The System of Neutrophil Elastase and Plasma Level of MMP-7 in Children with Pulmonary Arterial Hypertension and Chronic Cor Pulmonale

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Abstract

A significant increase in the activity of neutrophil elastase (NE) and anti-NE-protection in the plasma were detected in children having bronchopulmonary dysplasia (BPD) complicated by pulmonary arterial hypertension (PAH) and chronic cor pulmonale (CCP). The changes revealed were more pronounced in patients with CCP. The plasma concentration of the NE was slightly reduced, which was probably associated with the activation of anti-NE and an increase in the α1-antitrypsin level. A gradual increase was noted in the plasma level of the matrix metalloproteinase-7 (MMP-7) in patients with an increase in the severity of the condition. In patients with cystic fibrosis (with and without CCP), the pronounced increase in the MMP-7 level was observed. In patients with cystic fibrosis (CF), even without the additional complication with PAH and CCP, the MMP-7 level was significantly higher than in those with congenital broncho-pulmonary malformations (CBPM). The difference was increased in those patients with PAH and reached a maximum in those with CCP.

Keywords: *neutrophil elastase; matrix metalloproteinase-7; bronchopulmonary dysplasia; pulmonary arterial hypertension; chronic cor pulmonale.*

Introduction

According to modern concepts, pulmonary arterial hypertension (PAH) is a multifactorial pathology. The pathogenesis of PAH involves various biochemical processes and activities of different cells. The increased pulmonary vascular resistance is associated with several mechanisms including vasoconstriction, inflammation and thrombosis. In PAH, a reduction in the endothelium-derived vasodilators (nitric oxide, prostacyclin) and an increase in the thromboxane and endothelin-1 levels are evident. In the adventitia, an increase in the formation of the extracellular matrix components, including collagen, elastin, and fibronectin is also noted [1].

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Usually, the right ventricle (RV) operates under relatively low pressure and does not tolerate the PAH-induced overload. With the increase in the RV myocardial mass, the need for oxygenation is increased. Over time, in the enlarged and altered RV myocardium, dystrophic and necrotic changes keep occurring.

It is well known that the increased redox process and the development of oxidative stress (OS) are the pathogenetic mechanisms in the cardiovascular and bronchopulmonary pathologies, in the inflammations of any genesis. Structural membrane disruption by the OS activates the proteolytic enzymes, endonucleases, and NO synthase. Any impairment against the OS background may be caused by a change in the enzyme activity (or their direct inactivation or oxidative damage of the nucleic acids encoding them and the activities of the transcription factors) [2].

Processes occurring in the body during tissue metabolism have been developing with the involvement of various enzymatic systems and, chiefly, the proteolytic enzymes. It is believed that the action of the latter leads to a

reduction in the exudate viscosity and facilitates the removal of the heterogeneous respiratory products. Proteases influence the metabolism of the protein degradation products with the formation of assimilable amino acids. In a healthy lung, the proteases play an important role in the maintenance of homeostasis and regulate the regeneration and reparation processes. Chronic inflammatory lung diseases are associated with increased protease levels. Functionally, this can act positively against infection and inflammation, although only up to the point when the balance between the protective effects and destructive effects of the pulmonary proteases remains unbroken. The latter effects are associated not only with the protease activity, but also with the opposite controlling mechanism, namely with antiproteases. The main classes of the proteases present in the lung are serine, cysteine, aspartic, and metalloproteases. They can function within the cell as well as extracellularly. Neutrophil elastase (NE), the serine protease, plays the main role in this regulation. The NE can directly control the inducible expression and biological properties of the other pulmonary proteases. The action of these proteases is multifaceted, as follows: in inflammation, they involve the recruited cells and increase their migration; the NEs initiate an immune response and are involved in killing the bacteria, apoptosis, phagocytosis, as well as mucin production. However, how these non-destructive processes of the main pulmonary proteases are disconnected in pulmonary diseases continues to remain unclear [3,4].

There is evidence that the state of the myocardial stroma is regulated by the matrix metalloproteinases (MMRs) and their tissue inhibitors (TIMPs). In patients with chronic heart failure, the protease levels correlate with the degree of the left ventricular hypertrophy and fibrosis [5]. The MMP-7, also known as matrilysin, is a "minimal domain MMP". MMP-7 is synthesized and secreted in the intact exocrine and mucosal epithelium of most, if not all, adult tissues. The formation of MMP-7 in the normal epithelium suggests that this enzyme is involved in normal homeostatic functions. A series of studies emphasizes the role of matrilysin in the innate immunity of the epithelium. All the tissues, which express MMP-7, are open to bacterial infections and are easily damaged. Therefore, MMP-7 is the key regulator in the tissues exposed to the bacterial load, such as in patients with CF [6].

The aim was to study the complex changes in the phase of the protease - antiprotease balance in patients with BPD complicated by PAH and CCP.

Material and Methods

Between 2007 and 2011, we examined more than 240 children with various forms of BPD. In general, these patients had CBPM and CF; in addition, the study included children with idiopathic fibrosing alveolitis. Patients were divided into 3 groups: the 1st group included children with BPD without CCP and PAH (n=110); the 2nd group included children with BPD and PAH (n=80); the 3rd group included children with BPD complicated by PAH and CCP (n=52). As a control (the 4th group), data obtained from apparently healthy children without BPD were used (n=30). All the children were surveyed in the Scientific Center for Children's Health of RAMS. All children were about the same age, although patients with CCP were slightly older than those patients without PAH and CCP.

Blood was collected into tubes containing heparin-lithium. After 40 minutes of sedimentation, in the neutrophil-enriched plasma, the NE concentration and the anti-NE and anti-cathepsin G (anti-CG) activity were determined with ELISA using standard kits of "Bender Medsystems" and "Orgentic". The MMP-7 concentration was determined by ELISA using a standard kit of "Rand". The NE activity was determined spectrophotometrically (DU 530 spectrophotometer, Beckmann Coulter, USA) by measuring the breakdown intensity of N-metoxysuccinyl-ALA-ALA-PRO-VAL-L-Nitroanilide at λ 440 nm. The plasma levels of α1-antitrypsin (α1-AT) and α2-macroglobulin (α2-MG) were determined spectrophotometrically using the Sentinel kits (Italy); this method was adapted for the automatic analyzer "Synchron CX-5Δ" (Beckmann Coulter, USA).

Statistical analysis was performed using the statistical software "Statistica". The differences between groups were verified statistically, significant at probability forecasting ($p<0.05$). The correlation coefficient was computed by the Pearson method.

Results

As shown in Table 1, the NE level in BPD patients without PAH and CCP was not different from that in the control group. The PAH resulted in a significant increase in the NE level, while in the condition complicated by CCP a decrease in the NE level in the neutrophil-enriched plasma was observed. The NE levels in the patients of Groups 2 and 3 correlated with the α1-AT level (r=+0.49 and r=+0.6, respectively); at the same time, a negative correlation was revealed between the α1-AT concentrations and the anti-NE (r=-0.67 for Group 2 and r=-0.49 for Group 3) and anti-CG activities (r=-0.69 for Group 2). The NE activity in the BPD patients (Groups 1 and 2) was slightly increased compared with the control and significantly increased with CCP development. In Group 2 patients, correlations between the NE activity and degree of PAH were detected (r=+0.57). The increase in the NE activity was accompanied by an almost two-fold increase in the anti-CG activity. The anti-NE activity in the patients of Groups 1 and 2 was slightly lower and higher in the Group 3 patients when compared with the control. In the patients with CCP, the anti-NE activity was much higher than in the Group 1 patients. Significant differences were evident in the anti-NE activity between Group 2 and Group 3 patients. Similar changes were observed in the α1-AT level. In patients with CCP, a significant increase was noted in the α1-AT level compared with Groups 1 and 4. Noticeable fluctuations in the α2-MG level among the groups were not identified while a correlation between the α2-MG level and ages of the patients in Groups 1 and 2 was revealed (r=-0.64 and r=-0.61, respectively).

In light of these data, it was interesting to calculate the ratio of the amount of anti-NE and anti-CG antibody activities to NE activity. As evident from the Table 1, the anti-NE+anti-CG/NE activity ratio in Group 1 patients was slightly lower

than the control; in patients with the PAH development, this ratio gradually increases, but not significantly different from the control. In CCP patients, despite the increase in anti-NE activity and especially in the anti-CG activity, this ratio is drastically reduced, which indicates a complete failure of the adaptation processes in Group 3 patients in response to a sharp activation of the proteolytic enzymes. It is known that CG does not activate the hydrolysis of the synthetic substrate Succinyl-(L-ala)3-p-nitroanilide by elastase. We studied the activity of the NE on this substrate, to be able to get a complete picture of the NE activity. Thus, our results indicate that the CCP greatly increases both the NE activity and anti-NE activity; the plasma concentrations of NE slightly decreased, possibly as a result of the activation of the anti-NE protection and a change in the α1-AT level.

Table 1.
Protease –antiprotease imbalance in BPD patients

Parameters	Group 1	Group 2	Group 3	Croup 4
NE, U/ml	53.4±2.0	50.6±4.6	80.5±6.2 $p_{3/4}$<0.01	45.4±7.3
NE, ng/ml	119.3±5.7 $p_{1/4}$<0.001	189.2±18.4 $p_{2/4}$<0.01	159.4±16.8 $p_{2/3}$<0.01	127.5±13.5
Anti-NE, U/ml	307.5±11.7 $p_{1/3}$<0.001	319.5±23.7 $p_{2/3}$<0.01	431.0±20.7 $p_{3/4}$<0.01	331.4±18.0
Anti-CG, U/ml	423.0±14.6 $p_{1/4}$<0.01	338.4±16.1	510.6±25.1 $p_{3/4}$<0.001	264.0±49.9
Anti-NE+ anti-CG, U/ml	16.4±1.3 $p_{1/3}$<0.05	18.4±3.4	12.4±1.3 $p_{3/4}$<0.05	19.4±2.4
α_1-AT, g/l	1.66±0.09 $p_{1/3}$<0.05	1.75±0.17	2.35±0.27 $p_{3/4}$<0.05	1.62±0.04
α_2-MG, g/l	2.71±0.1	2.96±0.21	2.52±0.16	2.78±0.03
MMP-7, ng/ml	3.15±0.07 $p_{1/2}$<0.01 $p_{1/3}$<0.001	3.66±0.14 $p_{2/4}$<0.01 $p_{2/3}$<0.01	6.77±0.8 $p_{3/4}$<0.001	3.05±0.15
MMP-7, ng/ml (CBPM patients)	3.03±0.07	3.16±0.12	3.76±0.27 $p_{3/4}$<0.05	3.05±0.15
MMP-7, ng/ml (CF patients)	3.99±0.07	4.44±0.23	11.7±1.43	3.05±0.15
MMP-7 (CBPM+CF patients)	p_1<0.001	p_2<0.001	p_3<0.001	

We observed a gradual increase in the MMP-7 concentration in the BPD patients with the deteriorating condition and the development of PAH and CCP. Correlations were also detected between the MMP-7 concentrations and NE level (r=+0.51) and anti-CG activity (r=-0.44). In patients with CF, a pronounced increase in the MMP-7 level was observed. As revealed in the Table, in patients with CF, even without the additional complications of PAH and CCP, the MMP-7 level was significantly higher than in those with CBPM. The difference was seen to increase in patients with PAH and reached a maximum in those with CCP.

Discussion

Over the recent years, proteolysis has been recognized as a special form of physiological regulation. The proteolytic enzymes regulate the degradation of the abnormal and mutated protein structures and the formation and modification of the hormones, enzymes and physiologically active peptides. Proteolysis controls the concentration and quality of the main bio-regulators, the functioning of which determines the entire metabolism [7]. The azurophilic granules of the polymorphonuclear leukocytes contain two neutral serine proteases - NE and CG. The study of these enzymes is a rapidly evolving field of increasing interest due to their significance in the process of connective tissue degeneration.

It is known that some antibiotics specifically inhibit different steps in protein synthesis. The quantity of the enzyme (given the immunological cross-reactivity) varies a little, although it includes both the active and fully non-active molecules. Under the influence of the regulatory effects, many enzymes are transformed from an active form to an inactive form according to the metabolic needs of the cells. In general, NE plays a dual role. On the one hand, the NE can perform not only the intracellular destruction of the gram-positive bacteria captured by the neutrophils, it can also destroy extracellularly, and may possibly play a role in bacterial killing, being a part of the neutrophil extracellular traps (NETs). The NET substances are active against both gram-positive and gram-negative bacteria; they can destroy the virulence factors that come into contact with the NETs. In addition to the direct destruction of bacteria, the NE plays an important part in initiating inflammation and immunity, particularly, in the recruitment of the neutrophils and mucin gene expression [3]. The NE inactivates CD14 - a cellular receptor for lipopolysaccharides and reduces the inflammatory response to endotoxins. On the other hand, NE induces the expression of several cytokines, including the IL-8 production in the epithelial cells, destroys the phosphatidylserine receptors on the macrophage surfaces, thereby reducing their ability to remove the apoptotic cells [8]. The α1-AT exerts a similar influence, as well as an anti-inflammatory action. The simultaneous increase in the NE activity and the α1-AT level can probably be attributed to the compensatory response of the organism in patients with advanced CCP [9, 10].

NE and CG are defined in increased amounts during chronic inflammation. We observed an increase in the anti-CG activity among all the patients with BPD, particularly pronounced in Group 3 patients, which could be an indirect evidence of CG activation in these patients. We found a correlation between the magnitude of pulmonary pressure (PP)

and the anti-NE (r=+0.4) and anti-CG (r=+0.35) activities in Group 1 patients. In Group 2 patients, the PP correlated with the NE activity (r=+0.57). There is evidence that CG plays an important role in the case of low NE elastolytic activity against the pulmonary elastin; it is capable of activating the dissolution of the elastin by elastase, in 5-6 times. The polymorphonuclear leukocytes contain an equimolar amount of elastase and cathepsin G that leads to marked elastase activation [11].

In 1947, Grabar suggested that natural autoantibodies (NAs) are a part of the physiological mechanism for cleansing an organism of its own catabolic products. These have been found to play a role in protection against the infectious agents and to exert homeostatic functions.

The NAs are present in the circulation of normal human beings and other mammalian species that have not been exposed prior to deliberate immunization. They are often directed against highly conserved epitopes and often bind to the ligands of varying chemical compositions with low affinity. The NAs are frequently directed to intracellular structures, rather than to cell-surface antigens. The NAs are detected in normal human serum, against the antigenic structure of almost all organs and tissues. The NAs to the blood cells are also seen. In early '80s, it was found that the NAs to thyroxine interfered with the detection of the hormone by the radioimmunoassay method, giving inflated results. Perhaps, partly due to this, we identified an "inadequate" elevated NE level in Group 2 patients with respect to the NE activity [12].

It is known that, in the formation of the antimicrobial barrier and the excretion of the antimicrobial products, the epithelium provides the first line of defense against the invasive pathogens. Epithelial cells are recruited and limit the inflammation in the field of lesion. Matrilysin should not be seen as an example of proteinase catalyzing the matrix, but rather as an extracellular enzyme being involved in the regulation of cell-cell and cell-matrix signal pathways [13]. Synthesized in significant quantities in the adult tissues, MMP-7 is not detected during the fetal and early postnatal periods. Probably, the bacterial load is the physiological signal required to trigger the MMP-7 expression in the intact epithelium. The MMP-7 over-expression is observed in those patients with emphysema, idiopathic syndrome, pneumonia, transplantation and is most pronounced in the lungs of the CF patients. The bacteria themselves are indirectly the "substrate" for the MMP-7, and the presence of this substrate often regulates the production of all the MMPs. The matrilysin production is increased by 50 times in the epithelial tissue in contact with the bacteria although it is not potentiated by lipopolysaccharides [6].

It is interesting to note that the significant increase in the MMP-7 level in the CF patients was due to the addition of chronic viral and bacterial infections: the exacerbation of chronic sinusitis was seen in 4 patients, chronic hepatitis C in 2 patients; Mycobacterium tuberculosis, Staphylococcus aureus, and Pseudomonas aeruginosa were detected in many of the CF patients. This is consistent with the data of Winkler MK et al. (2003), who found that the MMP-7, MMP-8, and MMP-9 levels in the bronchoalveolar lavage fluid from sick children were significantly elevated compared with healthy individuals. It is known that in children with PAH, the timely treatment of respiratory tract infections is very important for protection from alveolar hypoxia, which occurs much more frequently in them than in adults. Any adverse effect on the lung tissue caused by the infections, toxic agents and pollutants increases the number of granulocytes and macrophages, which secrete the elastase. A significant increase in the proteolytic activity of the bronchoalveolar secretions leads to the destruction of the pulmonary tissue and primarily the elastic fibers. The inflammatory process itself can provide the persistence of various bacteria. For example, the human defensins may increase the adhesion of *H. influenzae* to the epithelial cells and thus predispose them to infestation. Currently, the interrelation between inflammation and fibrosis in the small airways has been revealed. Genetic differences may arise in response to the inflammation, proteolytic and protective mechanisms or susceptibility to latent viral infection.

The initially induced MMP-7 productions in the epithelium of the bronchi may subsequently be maintained with a low bacterial level. An interaction occurs between the NE and MMRs in the foci of the inflammation; in our study, the MMP-7 concentration correlated with the NE level in the CCP patients (r=+0,51). Proteases secreted by the epithelial cells may interact with each other, while maintaining the cycle of inflammation. A large number of MMRs (1,3,7,8,9,12) can cleave and inactivate the α1-AT; however, the NE, CG and proteinase-3 remain active. This may be important in the context of neutrophil activation and maintaining of the antimicrobial activity. In CF, even in the presence of NE, there is a reduction in the removal of the apoptotic cells from the respiratory tract, especially, if the NE splintered the phosphatidylserine receptors on the macrophage surfaces [4]. Phagocytosis of the apoptotic cells leads to their effective removal after the destruction of the plasma membrane and the release of the potentially dangerous intracellular contents. It is shown that the BAL of the CF patients contains 3 U/ml of NE and 28 mU/ml of CG. The increase in the anti-CG activity may indicate an increase in the content and/or activity of CG [15].

Conclusion

A significant increase in the activity of NE and anti-NE-protection in the plasma were detected in children having BPD complicated by PAH and CCP. The changes revealed were more pronounced in patients with CCP. The plasma concentration of the NE was slightly reduced, which was probably associated with the activation of anti-NE and an increase in the α1-antitrypsin level. A gradual increase was noted in the plasma level of the MMP-7 in patients with an increase in the severity of the condition. In patients with cystic fibrosis (with and without CCP), the pronounced increase in the MMP-7 level was observed. In patients with CF, even without the additional complication with PAH and CCP, the MMP-7 level was significantly higher than in those with CBPM. The difference was increased in those patients with PAH and reached a maximum in those with CCP.

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