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The activity of cathepsin D in saliva of cystic fibrosis patients

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Abstract: Cystic fibrosis (CF) is genetically determined illness, which is caused by the mutation in the CFTR gene. CFTR protein is also expressed in epithelial cells of parotid glands, therefore parotid glands are also affected in CF patients. Cathepsin D is one of the proteolitic cascade enzymes. Physiological wearing out result in occurrence of trace quantities of this enzyme in serum and body fluids, including saliva. Among different enzymes, saliva contains cathepsin D (CTSD, EC 3.4.23.5). The aim of this study was to determine cathepsin D activity in mixed saliva in cystic fibrosis patients and healthy controls. The study was performed in a group of 26 CF patients (10F, 16M). The results obtained in CF group was compared with the results of thirty healthy subjects (12F, 14M). From each subject 8 ml of mixed saliva was obtained: before and after the stimulation of saliva excretion using paraffin pledgets. Protein and glycoprotein content was assessed using Winzler's method. Protein concentration in controls and CF group before stimulation of excretion was 1.15±0.714 mg/mL and 1.54±0.925 mg/mL. After stimulation protein concentration in saliva has lowered to 0.88±0.77 mg/mL in CF group and 1.24±1.213 mg/mL in controls. Glycoprotein concentration in controls and in CF group was respectively: before stimulation 1.08±0.271 mg/mL and 1.05±0.344 mg/mL; after stimulation 0.92±0.292 mg/mL and 0.86±0.283 mg/mL. The activity of CTSD in controls was 45.9±24.98 Tyr nmol/mL/4h before stimulation and 109.3±56.94 Tyr nmol/mL/4h after stimulation of excretion. In CF group CTSD activity before stimulation was 134.5±81.80 Tyr nmol/mL/4h and after stimulation 134.4±62.18 Tyr nmol/mL/4h. Comparing the CTSD activity in both groups statistically significant difference has been revealed in samples collected before stimulation of excretion (p=0.013). The activity of cathepsin D in saliva of cystic fibrosis patient is significantly higher than in healthy controls before the stimulation of excretion with paraffin pledgets.

Key words: Cystic fibrosis - Cathepsin D - CTSD - Saliva

Introduction

Cystic fibrosis (CF) is genetically determined illness, which is caused by the mutation in the *CFTR* gene. CFTR protein, product of *CFTR* expression, acts as a cAMP dependent chloride channel in apical membrane of cells of respiratory epithelium and excretory ducts of glands. Defect of this ion channel in CF patients leads to impairment of excretion of chloride ions, and thus to retention of sodium and water absorption. As a consequence of this cascade, the viscosity of intrabronchiolar mucus is increased. The lumen of bronchi and internal organs is plugged by thick and dense mucus. Moreover, CFTR is also located in membranes separating cellular compartments. Impaired CFTR function can potentially alter enzyme activity in cells.

The digestion of food begins in oral cavity. The saliva provides proper, liquid environment for various biochemical processes. Among different enzymes, saliva contains hydrolases, including cathepsin D (CTSD, EC 3.4.23.5).

Cathepsin D is one of the proteolitic cascade enzymes. Physiological wearing out and decomposition of various tissues and cells result in occurrence of trace quantities of this enzyme in serum and body fluids, including saliva. Significant intensification of the damage and increase in CTSD activity in the blood takes place in certain pathological conditions such as breast cancer or uterine leiomyomas. According to the

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Table 1. Clinical characteristics of CF patients and controls.

	CF patients	Controls
Number of subjects	26	28
Females	12	15
Males	14	13
Age (yrs)	13.3±5.1	13.5±4.6

literature, elevated cathepsin D concentration and activity promotes *in vitro* the adhesion of *Pseudomonas aeruginosa* to corneal cells [15]. The aim of this study was to determine cathepsin D activity in mixed saliva in cystic fibrosis patients and healthy controls.

Materials and methods

Saliva. The study was performed in a group of 26 CF patients (10F, 16M). Clinical diagnosis of cystic fibrosis was confirmed by molecular examination of CFTR gene. The results obtained in CF group was compared with the results of thirty healthy subjects (12F, 14M). Clinical characteristics of both groups is showed in Table 1. Collection of mixed saliva were secured by standardized technique. From each subject 8 ml of mixed saliva was obtained. Then subjects chewed on 1.5 × 1.5 × 1.5 cm paraffin pledgets for 2 min. For next two minutes the mouth was rinsed with tap water to remove any residual paraffin and saliva. Then saliva was collected for the second time. The material was collected to sterile test-tubes and stored in -80°C until analysis.

Proteins and glycoproteins. Protein and glycoprotein content was assessed using Winzler's method [4]. Proteins and glycoproteins differ in solubility. Proteins in saliva were precipitated in reaction with chloric acid (VII). Glycoproteins in supernatant were precipitated with phosphorovolframic acid.

To 0.5 ml of whole saliva, 4.5 ml of 0.15 mol/L NaCl solution and 2.5 ml of 1,8 mol/L HClO4 The samples were incubated in room temperature for 10 min, then centrifugation was performed (at 4°C, 3000 rpm for 10 min). Supernatant was collected for glycoproteins measurement. Supernatant was dissolved in 1 ml of 10% Na_2CO_3, 3.5 ml H_2O and 0.5 ml of a Folin-Ciocalteau reagent. Samples were left for dying for additional 30 min. The absorbance was assessed at wave length of 750 nm. Protein concentration (C) was counted according to the following equation C=A*F (A - absorbance; F - calibration coefficient =4.62).

To 5 ml of supernatant, 1 ml of 5% phosphorovolframic acid was added. Samples were left for precipitation for 10 min, then were centrifuged (at 4°C, 3000 rpm for 10 min). After centrifugation supernatant was discarded and sediment was dissolved in 1 ml of 10% Na_2CO_3, 3.5 ml H_2O and 0.5 ml of Folin-Ciocalteau reagent. Samples were dyed after 30 min. The absorbance was assessed at wave length of 750 nm. The absorbance was measured at wave length of 750 nm. Glycoprotein concentration (C) was calculated according to following equation C=Tyr*F (Tyr - tyrosine concentration in mg/ml; A - absorbance × 800 = Tyr nmol/ml; F - calibration coefficient = 23.8).

Cathepsin D. The activity of CTSD was measured according to the following protocol. To 0.4 ml of saliva 0.1 ml of 6% hemoglobin (Disco Laboratories, Detroit, USA), denaturated with hydrochloric acid, was added and incubated for 6 h in 37°C. Reaction was conducted in pH=3.5 and terminated with 0.5 ml of 10% trichloracetic acid. After centrifugation (1500 g, 4 min, 0°C) the amount of released tyrosine was measured using the Folin-Ciocalteau method in the cupric modification [11]. Afterwards to 0.250 ml of supernatant 1.5 ml of cupric reagent was added (30 ml of 10% Na_2CO_3 and 1 ml of 0.5% $CuSO_4$ in 1% sodium citrate). After additional 10 min 0.250 ml of Folin-Ciocalteau reagent was added. The absorbance was measured after 30 min of incubation at wave length of 750 nm. The amount of released tyrosine was calculated according to following equation: C=A*F (C - tyrosine concentration in nmol/m, A - absorbance, F - calibration coefficient=800).

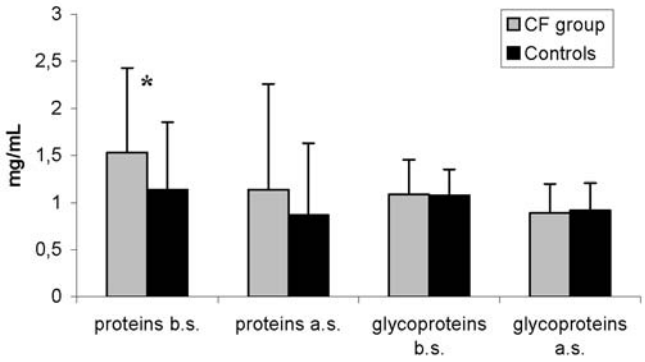

Fig. 1. Protein and glycoprotein content in saliva samples collected before stimulation (b.s.) and after stimulation (a.s.); *p≤0.05.

Ethical issues. The study was approved by the Local Bioethics Committee of Medical University of Bialystok. Each patient received detailed information about the aims and expected results of study and gave an informed consent.

Statistical analysis. Obtained results of enzymatic activity of both proteolytic enzymes were expressed to protein content in each specimen. The comparison of both studied and control group was performed using student's t test. For the statistical calculation a Sigma Stat 2.03 (SPSS Inc., Chicago, IL, USA) statistical package was used.

Results

Protein concentration

Protein concentration in controls and CF group before stimulation of excretion was 1.15±0.714 mg/mL and 1.54±0.925 mg/mL. After stimulation protein concentration in saliva has lowered to 0.88±0.77 mg/mL in CF group and 1.24±1.213 mg/mL in controls. The results in both groups are given in Table 2 and 3. Protein concentration was significantly higher in CF group than in controls (Fig. 1). After stimulation no such difference was observed.

Glycoprotein concentration

Glycoprotein concentration in controls and in CF group was respectively: before stimulation 1.08±0.271 mg/mL and 1.05±0.344 mg/mL; after stimulation 0.92±0.292 mg/mL and 0.86±0.283 mg/mL (Table 2 and 3). Differences in glycoprotein concentration before and after stimulation of saliva excretion in both groups were not statistically significant.

Table 2. Biochemical characteristics of saliva in healthy controls before stimulation (b.s.) and after stimulation (a.s.).

	Before stimulation	After stimulation	Significance (p value)
Protein content (mg/mL)	1.15±0.714	0.88±0.766	0.001
Glycoprotein content (mg/mL)	1.08±0.271	0.92±0.292	0.004
CTSD activity (Tyr nmol/mL/6h)	45.9±24.98	109.3±56.94	0.006
CTSD activity (Tyr nmol/1g of protein)	85.6±58.59	137.0±64.85	-
pH	6.4±0.62	7.0±0.43	0.541

Table 3. Biochemical characteristics of saliva in studied CF group before stimulation (b.s.) and after stimulation (a.s.).

	Before stimulation	After stimulation	Significance (p value)
Protein content (mg/mL)	1.54±0.925	1.24±1.213	0.014
Glycoprotein content (mg/mL)	1.05±0.344	0.86±0.283	0.724
CTSD activity (Tyr nmol/mL/6h)	134.5±81.80	134.4±62.18	0.092
CTSD activity (Tyr nmol/1g of protein)	127.1±29.84	161.7±61.65	-
pH	6.6±0.58	7.2±0.38	0.012

Activity of cathepsin D (CTSD)

The activity of CTSD in controls was 45.9±24.98 Tyr nmol/mL/4h before stimulation and 109.3±56.94 Tyr nmol/mL/4h after stimulation of excretion. Observed difference in CTSD activity before and after stimulation was significant (Table 2). In CF group CTSD activity before stimulation was 134.5±81.80 Tyr nmol/mL/4h and after stimulation 134.4±62.18 Tyr nmol/mL/4h. Comparing the CTSD activity in both groups statistically significant difference has been revealed in samples collected before stimulation of excretion (p=0.013). After stimulation the CTSD activity in both groups was similar (Fig. 2). The activity of cathepsin D expressed in nmol/1g of protein is presented in Fig. 3.

Discussion

The saliva creates appropriate environment in mouth for various physiologic processes. The composition of saliva depends of many factors. In CF patients the composition of saliva can be changed due to the presence of CFTR protein in epithelial cells of excretory ducts of salivary glands. Also the amount of saliva produced by CF patients can be diminished causing the symptoms of dry mouth and increasing the risk of den-

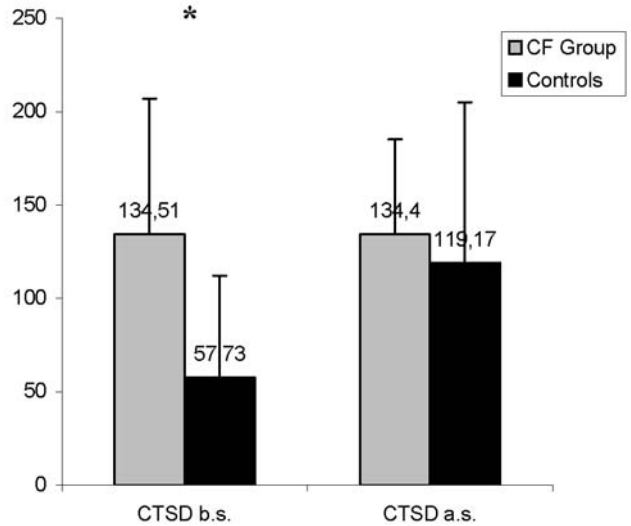

Fig. 2. Cathepsin D (CTSD) in nmol/mL/6h in saliva of CF patients and healthy controls before (b.s.) and after (a.s.) stimulation of excretion with paraffin pledgets; *p≤0.05.

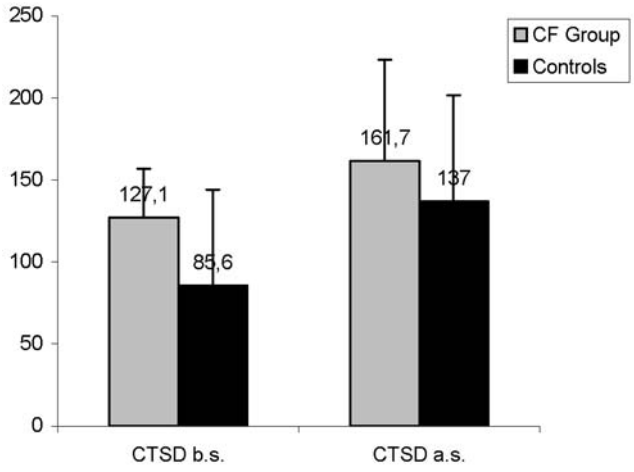

Fig. 3. Cathepsin D (CTSD) in nmol/1g of protein in saliva of CF patients and healthy controls before (b.s.) and after (a.s.) stimulation of excretion with paraffin pledgets; *p≤0.05.

tal caries [18]. CFTR is also found in membranes separating intracellular compartments of different pH [20]. Due to defective function of mutated CFTR, more alkaline pH is observed in Golgi apparatus, prelysosomes and endosomes. This causes the production of proteins with lowered number of sialic acid residues and increased number of sulphuric acid residues. Pseudomonas aeruginosa preferably adhere to glycoproteins deprived of sialic acid residues, therefore in CF patients we can observe increased colonization of lower respiratory tract with this pathogen. This is an immense clinical problem in these patients.

Cathepsin D is a recognized marker for breast cancer [16]. Overexpression of cathepsin D in breast cancer cells is well documented. For diagnostic and monitoring purposes cathepsin D levels can be measured in saliva [17]. Cathepsin D is also an endopeptidase found in lysosomes of all mammalian tissues. Primary function of this enzyme is cleavage of peptic bonds, but recently new interesting functions of it has been described [19]. Cathepsin D can be an important regulator of apoptosis acting as an direct activator of caspase 3 and 9. It can also stimulate the release of cytochrome c from mitochondrion and thus induce the intrinsic apoptotic pathway.

In this short communication we present our results showing, that the activity of cathepsin D in saliva of cystic fibrosis patient is significantly higher than in healthy controls before the stimulation of excretion with paraffin pledgets (Fig. 2). The difference is even more visible if the activity of cathepsin D is expressed in nmol per 1g of protein (Fig. 3). There is no data available concerning this problem in cystic fibrosis. Dong *et al*. in their study on mouse corneal cells infected with *P. aeruginosa* observed, that overexpression of cathepsins B, D and L is present [15]. Furthermore, an increase in CTSD mRNA was noted before mRNA for other cathepsins. Cathepsin D can also be involved in prolonged inflammatory response in lung and excessive apoptosis resulting in an activation of fibroblasts, and subsequently the induction of tissue remodeling with pulmonary fibrosis [21]. Our observations can be an interesting start point for further studies.

Acknowledgements

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