Determination of the concentration of cathepsin B by SPRI biosensor in children with appendicitis, and its correlation with proteasomes

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Abstract

Background. Cathepsin B (CatB) belongs to a family of lysosomal cysteine proteases and plays an important role in intracellular proteolysis.

Objectives. The concentration of CatB and 20S proteasome was evaluated in the serum of children with appendicitis, before and after surgery, on a basis of an innovative method for determining biomolecules concentration – surface plasmon resonance imaging (SPRI) biosensor.

Material and methods. Forty-two children with acute appendicitis, who were treated at the Department of Pediatric Surgery (Medical University of Bialystok, Poland), were randomly included into the study (age: 5–17 years, mean age: 11.5 ± 1 year). There were 15 girls and 27 boys in the study group. Eighteen healthy, age-matched subjects, admitted for planned surgeries, served as controls. Exclusion criteria were the following: severe preexisting infections, immunological or cardiovascular diseases that required long-term medication, and complicated cases of appendicitis with perforation of the appendix and/or peritonitis.

Results. The CatB concentrations in the blood plasma of patients with acute appendicitis were elevated before surgery, they were the highest 24 h after surgery, and were above the range of concentrations measured in controls; the difference was statistically significant. The CatB concentration measured 72 h after the operation was decreased, but still did not reach the normal range when compared with the concentration measured in controls (p < 0.05).

Conclusions. Cathepsin B concentration may reflect the metabolic response to acute state of inflammation, surgical intervention in the abdominal cavity and the process of gradual ebbing of the inflammation. The method of operation — classic open appendectomy or laparoscopic appendectomy — does not influence the general trend in the CatB concentration in children with appendicitis. There is a strong positive correlation between the CatB and 20S proteasome concentrations 24 h after surgery. The SPRI method can be successfully used for determining the concentration of active forms of enzymes presented in lysosomes in the diagnosis of inflammatory conditions in the abdominal cavity.

Key words: appendicitis, inflammation, proteasomes, cathepsin B, surface plasmon resonance imaging biosensor
Introduction

Cathepsins are a family of acidic endopeptidases, the activity of which is restricted to the lysosomal compartment under physiological conditions. Cathepsins take part in protein turnover by degrading unneeded proteins into amino acids. Various pathologic stresses, such as global cerebral ischemia, induce the release of cathepsins into the cytoplasm, where they perform their proteolytic function and can promote direct cell degradation. Cathepsin B (CatB) directly activates caspase-11 and/or caspase-1 pathways, e.g., in focal cerebral ischemia, and confirms that apoptosis, necrosis and autophagia are interrelated mechanisms that can act in synergy and lead to cell death in acute pathologic conditions. Cathepsin B is also responsible for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation through autophagy degradation of inhibitor of κBα (IκBα) in microglia and macrophages. The inhibition of CatB can rescue cells from tumor necrosis factor (TNF)-induced apoptosis. The inhibition of cathepsins leads to significant neuroprotection, because treatment with a specific CatB inhibitor significantly reduces infarct volumes.

Elevated levels of CatB in fibroblasts are typically observed in many chronic inflammatory diseases, including rheumatoid arthritis as well as periodontitis, because CatB promotes inflammation involved in the production of mature interleukin 1β (IL-1β). Interleukin 1β is a potent pro-inflammatory cytokine that is crucial for host-defense responses to infection and injury. Lysosomal membrane rupture and CatB activity are important for the activation of the NLRP3 inflammasome in response to some stimuli, where the inhibition of CatB attenuates IL-1β release. Cathepsin B also degrades collagens in fibroblasts and leads to tissue destruction.

Proteasome is generally considered a dominating component in protein degradation pathway. Through degrading regulatory proteins or their inhibitors, proteasome regulates many cellular transduction pathways. The ubiquitin–proteasome pathway also offers extremely high substrate specificity and the ability to quickly alter the rate of proteolysis. Extracellular proteasomes have been found to circulate in the plasma of patients suffering from a variety of inflammatory, autoimmune and neoplastic diseases. In various pathologic conditions, the concentration of circulating proteasomes correlates with disease activity. The release of proteasome in the serum may be a result of membrane disruption. Endothelial cells, vascular smooth muscle cells and tubular epithelial cells all release proteasome-active vesicles when injured in vitro. A study by Ito et al. demonstrated that extracellular 20S proteasome not only is released from cells, but also plays a functional role in physiological processes.

Using the surface plasmon resonance imaging (SPRI) biosensor, we wanted to determine the concentration of CatB in the diagnostics of inflammatory condition located in the abdominal cavity and its correlation with 20S proteasome.

Material and methods

Forty-two children with acute appendicitis, who were treated at the Department of Pediatric Surgery of Medical University of Białystok, Poland, between 2013 and 2014, were randomly included into the study (age 5–17 years, mean age 11.5 ±1 year). There were 15 girls and 27 boys in the study group. Twenty-six children were subjected to laparoscopic appendectomy, while 16 children were subjected to classic open appendectomy. Eighteen healthy, age-matched subjects, admitted for planned surgeries, served as controls. Exclusion criteria were: severe preexisting infections, immunological or cardiovascular diseases that required long-term medication, and complicated cases of appendicitis with perforation of the appendix and/or peritonitis. All parents of our patients gave written informed consent for both a clinical and biochemical follow-up. We did not note any postoperative complications in our patients after appendectomy. All patients were discharged home on the 3rd day after surgery in good general state.

Venous blood samples (1–2 mL) were drawn on admission, 24 h after the appendectomy and 72 h after the appendectomy. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes; the plasma was prepared according to standard protocols and was stored at −80°C. After all blood samples were collected and patient data recorded, the CatB and 20S proteasome concentrations were assessed using SPRI by the investigators blinded to other data.

Procedure of cathepsin B and 20S proteasome determination

The CatB and 20S proteasome concentration was determined using the SPRI biosensor. The exact description of the methodology of measurements and biosensor design was set out in previous papers. Gold chips were manufactured as described in other papers. The gold surface of the chip was covered with polyphotolar and hydrophobic paint.

Biosensor preparation

Chips were rinsed with ethanol and water, and then dried under a stream of nitrogen. They were then immersed in 20 mM of cysteamine ethanolic solutions for 2 h and, after rinsing with ethanol and water, dried again under a stream of nitrogen.

Surface plasmon resonance imaging measurements

Surface plasmon resonance imaging for the protein biosensor array was performed as described in another study.

Plasma samples from healthy children admitted for planned surgeries (n = 18) and from our patients after
appendectomy (n = 42) were diluted 2-fold with phosphate-buffered saline (PBS) (Biomed-Lublin, Lublin, Poland) and transferred onto the sensor surface for 10 min. The volume of the sample applied on each measuring field was 2 μL. The SPR1 technique allows sensitive determination of proteins using highly specific enzyme-inhibitor interactions. An immobilized cystatin C (potent inhibitor of lysosomal proteinases) was purchased from Sigma-Aldrich (St. Louis, USA) and used for the CatB entrapment on the biosensor surface. The biosensor construction and optimization of measurement conditions used were previously described.23 Briefly, plasma samples were placed directly on the prepared biosensor for ~10 min to allow interaction with the inhibitor (cystatin C). The biosensor was washed with water and HBS-ES buffer solution, pH = 7.4 (0.01 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 0.15 M sodium chloride, 0.005% Tween 20, 3 mM EDTA) (all Biomed-Lublin, Lublin, Poland) to remove unbound molecules from the surface. The SPR1 signal was measured twice on the basis of registered images, following the immobilization of cystatin C, and then following the interaction with CatB from the samples. The signal, which is proportional to coupled biomolecules, was obtained by calculating the difference between the signal prior to and following the interaction with biomolecules. The concentration was determined using the calibration curves of the SPR1 signal depending on the concentration of CatB (Calbiochem; Merck, Warszawa, Poland).

Statistics

The Mann-Whitney U test and the Kruskal-Wallis H test with Dunn’s post hoc correction to control for multiple testing were used to compare differences between groups. Statistical analyses were calculated with the STATISTICA PL (StatSoft Inc., Tulsa, USA) v. 10.0 program. A two-tailed p < 0.05 was considered significant. Correlations were examined by linear regression (r) using the Spearman’s test. We presented median concentrations of CatB and 20S proteasome in children with appendicitis before surgery, as well as 24 h and 72 h after the appendectomy.

Results

The CatB concentrations in the blood plasma of patients with acute appendicitis were elevated before surgery, they were the highest 24 h after surgery, and were above the range of concentrations measured in controls, with the difference being statistically significant. The CatB concentrations measured 72 h after the operation were low, but still did not reach the normal range when compared with the concentrations measured in controls (p < 0.05) (Fig. 1).

The 20S proteasome concentrations in the blood plasma of patients with acute appendicitis were the highest before surgery, and were above the range of concentrations measured in controls, with the difference being statistically significant. The 20S proteasome concentrations measured 24 h and 72 h after the operation slowly decreased over time, and still did not reach the normal range when compared with the concentrations measured in controls (p < 0.05) (Fig. 2). There was no statistical difference between the CatB and 20S proteasome concentrations in children operated on laparoscopically and in children after classic appendectomy (data not shown). There was a strong positive correlation between the CatB and 20S proteasome concentrations 24 h after the surgical intervention (r = 0.5871; p = 0.00004) (Fig. 3). Both proteasomal and lysosomal systems are responsible for proteolysis, which is more intensive under pathologic conditions, and,

![Fig. 1. Cathepsin B concentration in the plasma of children with acute appendicitis before (A), 24 h after (B) and 72 h after (C) appendicitis surgery, and in control group (Cg)]](image-url)
in this case, was induced by inflammation and enhanced by surgical intervention. There were no such correlations before and 72 h after the surgery (p > 0.05).

**Discussion**

Appendicitis is caused by a blockage of the hollow portion of the appendix, most commonly by a calcified “stone” made of feces, but also by inflamed lymphoid tissue from a viral infection, parasites or tumors. This blockage leads to increased pressure in the lumen of the appendix, decreased blood flow in the appendiceal wall and bacterial growth inside the appendix, causing inflammation. The combination of inflammation, reduced blood flow to the appendix and distention of the appendix causes tissue injury and tissue death.

Protein degradation occurs in response to the activation of intracellular signaling pathways that increase the activity of the ubiquitin-proteasome, calcium-induced calpains and apoptotic regulators, such as caspase-3. Various pathologic stresses induce the release of cathepsins – acidic endopeptidases, the activity of which is restricted to the lysosomal compartment – into the cytoplasm, where they perform their proteolytic function and can promote direct cell degradation.

Cathepsin B is released from lysosomes after the activation of specific apoptotic inducers, such as death receptors of the TNF family, transforming growth factor-beta 1 (TGF-β1), p53, and sphingosine, as well as during oxidative stress and starvation. Sphingosine induces lysosomal permeabilization in a CatB-dependent manner.

In our study, we found that the CatB concentrations in the blood plasma of patients with acute appendicitis were

such as arthritic pain and postoperative pain. 29

ful new strategy for treating inflammation-associated pain, roughly at that level for up to 2 weeks. 30 Importantly, the concentration in the abdominal cavity and the process of gradual ebbing of inflammation. We can conclude that lysosomal proteolysis connected with CatB activity was induced by inflammation and exacerbated by surgical intervention. Positive correlation between the CatB and proteasome concentrations 24 h after surgical intervention indicates higher destruction of cells and higher release of their content into the blood.

Apparentlly, the method of operation – classic open appendectomy or laparoscopic appendectomy – did not influence the general trend in the CatB and 20S proteasome concentrations in the blood of children with appendicitis. We may hypothesize that the resection of the appendix itself, regardless of the method of the operation, is the most influential factor in the process of reducing the inflammation resulting from acute appendicitis.

Cathepsin B regulates collagen expression by fibroblasts via prolonging toll-like receptor 2 (TLR2)/NF-kB activation during chronic inflammation and oxidative stress. 4 It is involved in degrading the intracellular and extracellular collagen produced by fibroblasts, thereby influencing the mechanisms of delayed tissue repair during chronic inflammation. Collagen type III and type IV in fibroblasts is involved in degrading the intracellular and extracellular space. Cathepsin B in the extracellular space can induce neuronal cell apoptotic death. Non-brain polytrauma patients show increases in plasma CatB activity during the 1st day after trauma, which subsequently falls to moderately elevated levels by the 3rd day and remains roughly at that level for up to 2 weeks. 30 Importantly, the increase in plasma CatB activity correlates with the severity of injury. 30–32 Deleting or inhibiting CatB improves outcomes in injury models related to TBI, including epilepsy, aneurysm, ischemia, pain, surgical trauma, spinal cord trauma, infectious disease, and neurodegeneration. 30

Cathepsin B is also a candidate target for inhibiting hepatocyte apoptosis in liver diseases. 33 Both genetic and pharmacologic inactivation of CatB reduces liver injury, inflammation and hepatic fibrogenesis during cholestasis. 24 Cathepsin B-mediated liver injury not only causes apoptosis, but also stimulates the production of proinflammatory chemokines. 33 Furthermore, inhibiting CatB catalytic activity with selective protease inhibitors, such as R-3032, might be a potential therapeutic option for cholestatic liver injury, inflammation and fibrosis. 33

Cathepsin B is a potential drug target for several diseases, including various cancers, pancreatitis, liver fibrosis, rheumatoid arthritis, viral Ebola, bacterial Streptococcus pneumoniae meningitis, TBI, and parasitic Trypanosoma cruzi infections. 5,30,34–43 We strongly believe that further studies on CatB and its relation with inflammation located in the abdominal cavity and surgical interventions may bring future clinical implications.

Conclusion

Cathepsin B concentration may reflect the metabolic response to acute state of inflammation, surgical intervention in the abdominal cavity and the process of gradual ebbing of inflammation. The method of operation – classic open appendectomy or laparoscopic appendectomy – does not influence the general trend in the CatB concentration in children with appendicitis. There is a strong positive correlation between the CatB and 20S proteasome concentrations 24 h after surgery. Biosensors with SPRi as a detecting method can be successfully used for determining the concentration of active forms of enzymes presented in lysosomes in the diagnostics of inflammatory condition in the abdominal cavity.

References


