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Determination of cathepsin G in endometrial tissue using a surface plasmon resonance imaging biosensor with tailored phosphonic inhibitor

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ABSTRACT

Objective: Cathepsin G is a serine peptidase whose physiological role is mainly associated with an early immune response, anti-microbial activity as well as platelet activation or hydrolysis of coagulation factors. In addition, since the activity of cathepsin G has been associated with the development of various pathological disorders, the measurement of its activity in patient samples is of high interest. Unfortunately, the usefulness of common immunological methods is limited, since they cannot distinguish between catalytically active and inactive protease.
Study design: Here we present the application of recently developed Surface Plasmon Resonance-based biosensor for the detection of active cathepsin G in human endometrium samples. The key element of the system is based on the irreversible binding of cathepsin G to its specific phosphonic-type inhibitor immobilized on the surface of the gold chip. The concentration of cathepsin G was measured in tissue samples from the group of patients with endometriosis as well as in the control group.
Results: The level of cathepsin G ascertained in endometrium tissue samples was over twice as high for the group of patients suffering from endometriosis as compared to the control group, with the median values of 0.5 pmol/mg and 0.2 pmol/mg, respectively.
Conclusion: The SPR sensor armed with a specific irreversible phosphonic inhibitor represents a highly useful tool for the determination of catalytically active cathepsin G concentration in endometrial tissue.

Introduction

Cathepsin G (CatG, EC 3.4.21.20) belongs to the serine protease family and is expressed mainly by neutrophils, monocytes as well as mast and dendritic cells. The protease is stored in azurophil granules and upon stimulation is released into the extracellular environment. The physiological role of CatG is associated with an early immune response, anti-microbial activity [1,2] as well as platelet activation [3] or hydrolysis of coagulation factors [4]. A fraction of CatG remains associated with cell membrane and can contribute to an inflammation process although differently from the soluble form, since the membrane-bound form is resistant to inhibition by endogenous serpins [5,6]. The increased CatG activity has been associated with several pathological disorders such as rheumatoid arthritis or lung diseases [7]. Despite the fact that CatG plays an important role in various biological pathways, its complete function has not been fully understood, and the analytical methods for the determination of its active form are not satisfactory for diagnostic applications.

Endometriosis is defined as a chronic gynecological disorder affecting approximately 6–10% of women, and is described as an abnormal localization of endometrial tissue outside the uterus [8]. Despite its benign progression, it is considered as a potential risk factor in the development of the ovarian cancer [9]. Due to the inflammatory character of endometriosis, there are several indications of the existence of a disturbed immunological status

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of endometriosis foci, eutopic endometrium as well as peritoneal fluid. This is reflected by an altered status of different biologically active substances such as chemokines, angiogenic factors, matrix metalloproteinases, tumor suppressor genes, microRNAs and potentially many others [10–14].

Additionally, a study using a mouse model showed macrophage stimulation and infiltration of neutrophils into the peritoneal fluid after the implantation of uterine tissue into the peritoneum [15]. A further analysis of peritoneal fluid of patients with endometriosis has demonstrated an increased level of cathepsin D [16], human neutrophil peptides 1–3 [17], cathepsin G [18] or neutrophil elastase [19], which indicates the involvement of neutrophils in the process of endometriosis development. Although the role of neutrophils in pathogenesis of endometrial cells is still unknown, it has been suggested that an increased neutrophil-to-lymphocyte ratio may present the diagnostic value for endometriosis [20].

The quantification of protein levels in human samples allows for a better understanding of the proteome, which, in case of pathological stages, can provide valuable information for the discovery of potential novel diagnostic markers or drug target. However, although most of commonly applied antibody-based immunological techniques have been found extremely useful in disease diagnostics including cancer and infections, the detection of enzymatically active proteases requires a slightly different approach, since an immunological approach cannot distinguish between active and inactive species. The surface plasmon resonance imaging (SPRI) technique together with suitably designed biosensors allows for a sensitive and specific detection of a target biomolecule: an immobilization of a selective enzyme inhibitor on the surface of the gold chip allows for a rapid quantification of the active enzyme form present in the sample [21–28].

Until now several synthetic inhibitors of CatG have been developed. One of the most interesting groups of serine protease inhibitors are α-aminoalkylphosphonate diaryl esters, which are analogues of amino acids reacting irreversibly with the active site of the target protease. Due to their structural resemblance to the transition state observed during peptide bond hydrolysis and to the presence of the electrophilic phosphorus atom, aromatic esters of α-aminoalkylphosphonic acids display a high potency of action toward target serine protease exclusively, lacking any reactivity with cysteine, threonine or metalloproteases. Moreover, even for two serine proteases that share similar substrate recognition pattern almost absolute specificity can be achieved by the modifications of the P1 group (Schechter and Berger nomenclature [29]), an elongation of the peptidyl chain or through an introduction of various electron-withdrawing groups into *para* position of the phenyl ester ring [30–32].

In our previous study we ascertained that the concentration of cathepsin G was higher in eutopic endometrium collected from patients with early endometriosis (stages I and II) as compared to a control group [18]. Herein we present the application of recently developed SPR-based biosensor containing a selective phosphonic inhibitor for the determination of active CatG in endometrium samples, both from patients suffering from early endometriosis and from healthy individuals. In addition, through the application of molecular modeling methods, the inhibitor was docked into the CatG active site, giving an insight into the interaction network formed between both partners of the complex.

Materials and methods

Reagents, materials, methods

Cathepsin G from human sputum, all reagents and solvents for α-aminoalkylphopshonate synthesis, cysteamine, and Tween 20 were purchased from Sigma-Aldrich (Poznań, Poland). Salts and solvents used for buffers preparation were from POCh (Gliwice, Poland) and Biomed (Lublin, Poland). Photopolimer ELPEMER SD 2054 and hydrophobic protective paint SD 2368 UV SG–DG were from PETERS (Kempen, Germany). All reagents and solvents used for the solid phase peptide synthesis were purchased from IRIS Biotech (Marktredwitz, Germany). Aqueous solutions were prepared with milliQ water (Simplicity®, Millipore, Poland). Endometrial tissues were provided by the Department of Perinatology of Medical University in Bialystok, Poland. All samples were provided after obtaining the consent of the Bioethical Commission.

Molecular modeling—The inhibitor docking

Flexible ligand docking was performed using AutoDock Vina 1.1.1 [33]. CatG crystal structure coordinates were chosen from the Protein Data Bank (1AU8.pdb). The water molecules and the ligand were removed from the protein structure. MARS-115 structure was used in a phosphonic acid form in order to demonstrate an enzyme-inhibitor aged complex. The polar hydrogen atoms were added, using AutoDockTools 1.5.4 (Scripps Research Institute, USA). The size of a grid box (surrounding CatG active center and binding pockets) in each dimension was 22 Å.

CatG inhibitor synthesis

The preparation of the CatG inhibitor (MARS-115) started with the synthesis of *t*Bu-Suc-Phe-Val-Thr(*O*-*t*Bu)-OH on solid support, using an Fmoc strategy [34]. For the synthesis of the phosphonic component of the inhibitor, an amidoalkylation reaction was applied [35–37]. Finally, the inhibitor was purified by HPLC (Varian ProStar 210 instrument equipped with a C_8 column, 10 × 250 mm, 5 μm, SunFire™ Prep) to homogeneity. The molecular weight of MARS-115 was further confirmed by high resolution mass spectrometry (Waters Acquity Ultra Performance LC, LCT Premier XE) yielding: *m/z* $[M + 1]^+$ 936.3140 (observed); 936.3190 (calculated).

Chip preparation

Gold chips were manufactured as described previously [26,27,38]. The obtained array of 9 × 12 free gold surfaces was covered with a photopolymer and a hydrophobic paint in such a way that 12 free gold spots of each measuring field were separated by photopolymer, while 9 measuring fields were separated with hydrophobic paint, allowing for simultaneous measurement of nine different samples with twelve replications of SPRI measurements for each sample tested. Immobilization of MARS-115 and conditions optimization, SPRI measurements and standard curve preparation were performed as described previously [39].

Preparation of endometrial samples

Endometrial samples were obtained from regularly menstruating premenopausal women aged 20–35, undergoing diagnostic laparoscopy. Patients with minimal and mild peritoneal endometriosis, stages from I to II, were diagnosed by laparoscopic findings according to the revised AFS classification of endometriosis [40], and each case was confirmed by histopathology.

Patients with autoimmune disease, pelvic inflammatory disease, adenomyosis, dysfunctional uterine bleeding and those who took non-steroidal anti-inflammatory drugs, GnRH agonists and steroids for the past 3 months were excluded from the study. Endometrial control samples were collected by Pipelle suction in the operating room before the laparoscopic procedure and endometriosis samples were taken during laparoscopy. Endometrial tissue samples were classified with histological dating according to the method of Noyes

et al. [41] and only patients in the proliferative phase (days from 8th to 12th) of the cycle were included in the study.

All proteins from freshly frozen endometrial tissue were extracted by means of a mirVana™ PARIS™ Kit (Ambion® by Life Technology, Warsaw, Poland) following the manufacturer's protocol. The samples were diluted 50-times with buffer (pH 8.0), transferred onto the sensor surface (4 μl per each measuring field) and incubated for 10 min. The biosensor surface was then washed 10-times with the HBS-ES buffer and 10-times with water. After the SPRI measurement, the CatG concentration was calculated with the prepared calibration curve. The results were expressed in pmol of CatG per mg of total protein present in the endometrial sample. The results for each endometrial sample were obtained from 12 independent measurements, and presented as an arithmetic mean with standard deviation.

Results

Development of inhibitor

α-Aminoalkylphosphonate diaryl esters constitute a family of specific, irreversible inhibitors of serine proteases. The mechanism of their action relies on the nucleophilic attack of the active site serine on the phosphorus electrophilic center, thus forming a covalent enzyme-inhibitor complex. Although simple, Cbz-protected aminophosphonates display a relatively low specificity and selectivity toward target protease, a high degree of reactivity can be obtained *via* introduction of the N-terminal peptidyl chain, which allows for the formation of a close, optimal interaction network between the protease and inhibitor molecule occupying its binding pockets. On the basis of the substrate specificity of CatG, a highly potent and selective phosphonic inhibitor has been recently developed [42]. An introduction of a terminal carboxylic function allowed for its covalent binding to the surface of the biosensor chip [27]. The heterobifunctional character of the inhibitor enabled the development of SPRI biosensor for the determination of active CatG in biological samples: the terminal carboxylic group was covalently bound to the chip surface via a thiol linker, while the phosphonic warhead reacted irreversibly with the protease active site.

CatG–inhibitor interaction analysis

In order to get an insight of MARS-115 interaction with CatG, we performed a docking simulation of the inhibitor within the CatG binding pockets (Fig. 1A). The structure with the best docking score was selected as the binding mode of the inhibitor. The P1 side chain of the inhibitor molecule fills the S1 pocket, where the *p*-guanidine group interacts with the carboxylic side chain of Glu226 located at its bottom, which is crucial for a specific recognition and directs the inhibitor molecule in close proximity to become irreversibly bound by the catalytic serine oxygen (Fig. 1B). Residues P2, P3 and P4 of the inhibitor are located in the corresponding S2–S4 binding pockets, whereas the succinyl chain is shifted close to the protein surface (Fig. 1A and C). Such orientation of the terminal succinyl group allows for the specific binding of CatG to the inhibitor attached to the chip surface. The superposition of MARS-115 with *N*-(3-carboxypropanoyl)-L-valyl-*N*-[(1*R*)-5-amino-1-phosphonopentyl]-L-prolinamide present in the crystal structure of CatG used for docking simulation (1AU8.pdb) is shown on Fig. 1D.

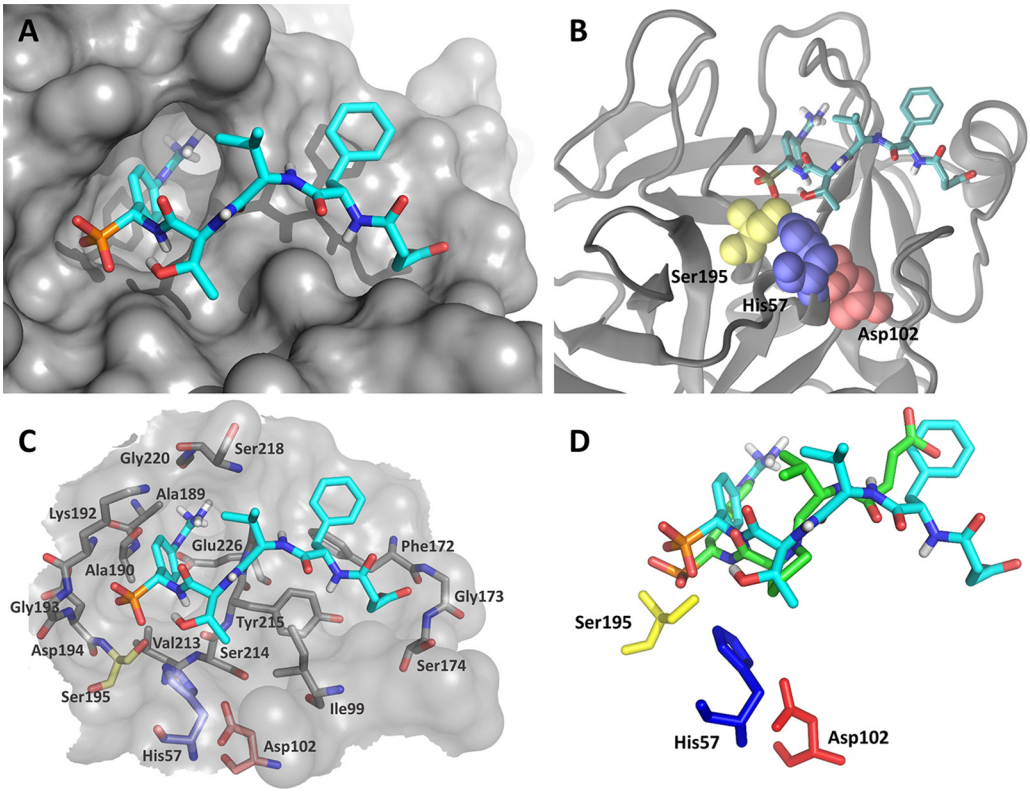

Fig. 1. Model of MARS-115 docked in the CatG active site. (A) The structure with the best docking score in the CatG binding site. (B) Ribbon representation of CatG with MARS-115 docked within the active site with spherical representation of catalytic triad residues. (C) CatG residues surrounding the inhibitor molecule that may be involved in its binding. (D) Superposition of MARS-115 and *N*-(3-carboxypropanoyl)-L-valyl-*N*-[(1*R*)-5-amino-1-phosphonopentyl]-L-prolinamide and their orientation toward CatG catalytic triad residues.

Table 1
Cathepsin G concentration in endometrium tissue samples. The results are presented as mean ± SEM and represents 12 independent measurements performed for each sample.

Control samples			Endometriosis samples		
Sample number	C_{CatG} [pmol/ml]	C_{CatG} [pmol/mg]	Sample number	C_{CatG} [pmol/ml]	C_{CatG} [pmol/mg]
1	1.1 ± 0.4	0.2 ± 0.1	1	2.6 ± 0.6	0.5 ± 0.1
2	1.1 ± 0.5	0.4 ± 0.2	2	2.7 ± 0.4	0.6 ± 0.1
3	0.9 ± 0.2	0.2 ± 0.01	3	2.8 ± 0.5	0.6 ± 0.1
4	0.9 ± 0.4	0.3 ± 0.1	4	2.6 ± 0.5	0.6 ± 0.1
5	1.1 ± 0.4	0.2 ± 0.1	5	2.2 ± 0.4	0.5 ± 0.1
6	1.6 ± 0.5	0.3 ± 0.1	6	2.5 ± 0.5	0.6 ± 0.1
7	1.1 ± 0.5	0.4 ± 0.2	7	2.5 ± 0.5	0.5 ± 0.1
8	1.2 ± 0.7	0.3 ± 0.2	8	2.3 ± 0.5	0.5 ± 0.1
9	1.3 ± 0.4	0.2 ± 0.1	9	2.3 ± 0.5	0.4 ± 0.1
10	1.0 ± 0.6	0.2 ± 0.1	10	2.3 ± 0.5	0.5 ± 0.1
11	1.0 ± 0.5	0.2 ± 0.1	11	2.4 ± 0.4	0.5 ± 0.1
12	1.0 ± 0.4	0.2 ± 0.1	12	2.4 ± 0.4	0.6 ± 0.1
13	1.3 ± 0.4	0.2 ± 0.1	13	2.5 ± 0.5	0.7 ± 0.1
Median	1.1	0.20	Median	2.5	0.5
Range	0.9–1.3	0.2–0.4	Range	2.2–2.8	0.4–0.7

Characteristics of the biosensor

Detailed analytical characteristics of the biosensor have been published elsewhere [28]. Briefly, the dependence of the analytical signal under the optimized conditions was investigated within a CatG range of 0.25–2.5 ng/ml. Within a range of 0.25–1.25 the calibration curve was linear ($y = -463 + 3997x$ with $R^2 = 0.9903$, where y is the analytical signal (arbitrary units) and x is the enzyme concentration ng/ml). Chymotrypsin, human neutrophil elastase and porcine pancreatic elastase did not interfere with CatG determination, and neither did subtilisin, proteinase 3 and trypsin at the same concentration as CatG.

Determination of CatG in endometrial tissue samples

Endometrial tissue samples were processed as described above and analyzed using the SPRI biosensor. The group of 13 samples from patients suffering minimal and mild endometriosis (stages I and II, $n = 13$), as well as 13 samples of healthy individuals were investigated. The obtained results are presented in Table 1. The concentration of CatG in tested samples is expressed in pmol/ml and pmol/mg of the total protein present in the sample. The results are coherent and show that the CatG concentration in endometrial tissue of patients suffering endometriosis is more than twice as high as compared to the control group with median values of 0.5 pmol/mg (range 0.4–0.7 pmol/mg) and 0.2 pmol/mg (range 0.2–0.4 pmol/mg), respectively ($p = 0.05$). The elevated level of CatG in endometriosis is most likely the consequence of increased neutrophil counts as reported previously [20]. The results may also highlight the applicability of the SPRI sensor as a potential diagnostic tool for the detection of endometriosis.

Comments

Herein we present the application of recently developed SPRI biosensor based on a highly potent CatG inhibitor for the determination of CatG in endometrial tissue. The inhibitor was modified with a terminal carboxylic function in order to facilitate its covalent binding to the chip surface. The high specificity of the SPRI sensor resulting from the interaction of the core inhibitor immobilized onto the chip surface, and active CatG present in the sample allows for the determination of CatG in a picomolar concentration range. To the best of our knowledge no other technology allows for such sensitive detection of enzymatically active CatG. Moreover, the SPRI technology based on the application of specific an irreversible phosphonic inhibitor distinguishes between active-inactive forms of CatG, which is not possible for commonly used immunostaining approaches, thus giving more valuable predictive information regarding the proteolytic activity in the sample.

Since previous studies have indicated the involvement of immune system cells in the development of endometriosis, we have focused on the detection of neutrophil-derived CatG in the endometriosis tissue. In our study we have shown the elevated concentration of the active cathepsin G in endometrial tissue samples. The level of CatG in patient samples was more than twice as high as compared to control samples. The developed SPRI array biosensor presents a highly specific method for the determination of active CatG with potential diagnostic applicability. In addition, since the applied phosphonic inhibitor irreversibly binds to the active site of CatG, thus it limits the possibility of CatG liberation from the complex and escaping the detection.

Condensation

The article presents the application of the Surface Plasmon Resonance-based biosensor for the detection of active cathepsin G in endometrial tissue samples.

Conflict of interest statement

The authors report no conflict of interest.

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