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Characterization of potential elastase inhibitor-peptides regulated by a molecular switch for wound dressings applications

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ABSTRACT

Elastase plays an important role in wound healing process, degrading damaged tissue and allowing complete tissue recovery. The levels of human neutrophil elastase (HNE) are usually controlled by endogenous inhibitors. However, in the presence of high levels of elastase, like the ones present in chronic wounds, the inhibitors cannot overcome this overproduction and the enzyme starts to degrade the surrounding healthy tissue. In this work we report the development of a molecular switch to control the elastase activity in the exudate of non-healing chronic wounds. A peptide library was generated and screened in a microarray format for protein kinase-mediated phosphorylation. Two peptides were identified as casein kinase Iδ (CKI) substrates: KRCCPDTCGIKCL and its analogous peptide KRMMPDTMGIKML, with cysteine residues replaced by methionine residues. These peptides were studied in solution, both in the phosphorylated and non-phosphorylated forms as potential inhibitors for elastase. The obtained results show that the reversible process of phosphorylation/dephosphorylation results in differential inhibitory activity of the peptides. Thus the reversible process of phosphorylation/dephosphorylation can be used as a kind of molecular switch to control elastase activity. Degradation studies reveal that both the inhibitor-peptides and CKI are degraded by elastase. These results envisage the safe utilisation of these inhibitor-peptides together with CKI in the formulation of wound dressings.

1. Introduction

Non-healing chronic wounds, such as pressure, venous and diabetic ulcers, are an important and persistent problem in dermatology [1]. In healing acute wounds such as injuries or surgical procedures (fluids from ablation of seborrheic warts, for example) the levels of protein-degrading enzymes are low whereas in chronic non-healing wounds the exudates (from ulcers fluids, for example) contain high levels of proteases, such as elastase and matrix metalloproteinases (MMPs) [2–4]. Elevated levels of elastase (36–54 munits/mL wound fluid) [5] degrade cytokine growth factors, fibronectin and reduce the endogenous levels of protease inhibitors. In acute wounds, minimal levels of elastase (245.9 ng/mg protein) [4] and matrix metalloproteinases (1.6 ng/mg protein MMP-2 and 0.2 ng/mg protein MMP-13) [4] are required for an appropriate healing process. The elastase levels are controlled by endogenous inhibitors such as alpha-1 proteinase inhibitor (α1-PI), secretory leukocyte protease inhibitor (SLPI) and Elafin [6].

The levels of proteases in chronic wounds may be reduced to levels similar to those found in acute wounds using bioactive wound dressings composed of peptide [7,8] and carbohydrate derivatized cotton [9], ionically derivatized dressings of cotton [10,11] and hydrogel polymers [12]. Controlled release of protease inhibitors from wound dressings and biomaterials, such as collagen, alginate, chitosan, carboxymethylcellulose, hydrogel polymers, hydrocolloids and polyurethane [13,14] have also been used to reduce the protease burden on chronic wounds. Sequestration of elastase from the wound environment [11,12,15] or the release of elastase inhibitors to the wound medium [5,16], are two plausible approaches to control elastase levels in wound exudates. The present work relies on the second concept as a methodology to control the imbalance between proteases and their inhibitors. The inhibitor-peptides studied herein were selected from the endogenous elastase inhibitors SLPI and Elafin and from two other endogenous proteins, eosinophil cationic protein (ECP) and surfactant protein D (SP-D). Elafin and SLPI have high cysteine content,

Abbreviations: SLPI, secretory leukocyte protease inhibitor; ECP, eosinophil cationic protein; SP-D, surfactant protein D; ESI, elastase specific inhibitor; HNE, human neutrophil elastase; PPE, porcine pancreatic elastase; MBP, myelin basic protein; CKIδ, casein kinase I delta isoform; Ser(P), phoshoseryl residue; Thr(P), phosphothreonyl residue; Xaa, any given aminoacid; HTS, high throughput screen; RLUs, relative light units; EC_{50}, enzyme concentration at 50% of signal; Peptide 4, Pep4; Peptide 4, KRCCPDTCGIKCL; Peptide 4 Modified, Pep4M; Peptide 4 Modified, KRMMPDTMGIKML; p-NA, *p*-nitroaniline; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SD, standard deviation.

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8 and 16 residues respectively, with the correct pairing of disulfide bridges being crucial for elastase inhibition [17–19]. Elafin is a potent inhibitor of both human neutrophil elastase (HNE) and porcine pancreatic elastase (PPE) [18–20], whereas SLPI is a strong HNE inhibitor but a weak PPE inhibitor [18–20]. SLPI and Elafin have multiple biological functions: anti-bacterial, anti-fungal, anti-viral, anti-inflammatory and immune-modulatory functions [20–22]. Eosinophil cationic protein is a potent stimulator of mucus secretions by airways epithelial cells [23] acting as a host defence protein due to its bactericidal, helminthotoxic and antiviral activities [24–26]. Furthermore, ECP displays tissue-healing properties regulating fibroblast activity and enhancing collagen release [23,27]. Surfactant protein D (SP-D), originally described as a collagenous glycoprotein [28], belongs to the collectin family of proteins, named for their N-terminal collagen region and C-terminal lectin domain. SP-D is a large hydrophilic molecule with host defence and immune regulatory functions [29–31]: viral neutralization, clearance of bacteria, fungi and apoptotic and necrotic cells, down regulation of allergic reaction and resolution of inflammation [31].

Synthetic protease inhibitors are typically used for protease inhibition. Continuous release of (non-degradable) inhibitors from wound dressings results, over time, in steady concentration build-up in wounds, lowering protease activity levels below those desirable for healing. *In vivo*, the activity of endogenous proteases is controlled by small protein molecules (inhibitors), known as anti-proteases. A dynamic state of inhibition is achieved by regulating the relative rates of inhibitor synthesis and protease-mediated inhibitor degradation.

We envisage that steady-state low levels of elastase activity in chronic wounds can be achieved by sustained release of (degradable) inhibitor-peptides from wound dressings, coupled to elastase-mediated hydrolysis (deactivation). In addition, if the phosphorylated form of the inhibitor-peptides inhibit elastase to a less extent than the non-phosphorylated form, than protein kinase-mediated phosphorylation reaction, can be seen as a (0–1) molecular switch to fine tune elastase activity.

In the current work we give the first steps towards the proof-of-concept of this proposal: (i) a peptide library was designed using short peptide sequences derived from the endogenous proteins SLPI, Elafin, ECP and SP-D; (ii) all library members contain a central phosphorylable residue, Ser, Thr or Tyr; (iii) a library of 49 peptide sequences was screened in microarray format for phosphorylable peptides; (iv) the most robust hit identified in the microarray assay, Pep4, was selected for further studies; (v) the phosphorylation of Pep4 and its analogue Pep4M with casein kinase Iδ (CKI) was studied in solution; (vi) the inhibition of elastase by Pep4, Pep4M and their phosphorylated forms was evaluated in solution; (vii) the degradation of Pep4, Pep4M and CKI by elastase was studied in solution by mass spectrometry (MS) and SDS-PAGE electrophoresis, respectively.

2. Materials and methods

2.1. Reagents

The microarrays and the peptides KR**CC**PDT**C**GIK**C**L (peptide 4 – Pep4) and KR**MM**PDT**M**GIK**M**L (peptide 4 modified – Pep4M) were custom-made by JPT Peptide Technologies GmbH (Berlin, Germany). Except where otherwise stated, all reagents were purchased from Sigma Co (St. Louis, MO, USA). Casein kinase Iδ (C4455), Adenosine 5′-triphosphate disodium salt (ATP, A2383) and all reagents used to prepare the buffer solutions employed in the microarray assays, were used as supplied. The microarrays chips were sealed with disposable incubation chambers (AB-0630, 300 μL, 19 × 60 mm, frames and coverslips, ABgene, Epsom, UK). The blocking reagent (cat n°11096176001) used in the preparation of the blocking buffer was acquired at Roche Diagnostics GMbH (Basel, CH). The microarray stain and destain were performed using the Pro-Q diamond phosphoprotein gel stain (P33301) and Pro-Q diamond destain solution (P33310) from Molecular Probes (Eugene, OR, USA).

The protein kinase assay in solution was performed using the PKLight^R High Throughput Screen Protein Kinase assay Kit (LT07-500), from Cambrex (East Rutherford, NY, USA), in a 96-well format (F96 NUNC, Rochester, NY, USA). This assay kit comprises ATP detection reagent (LT27-200), reconstitution buffer A (LT27-202) and B (LT27-207) and kinase stop solution (LT27-228).

Porcine pancreatic elastase (E1250), chromogenic substrate *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (S4760) and the non-peptide PPE inhibitor Elastatinal (BML-PI-103, Enzo Life Sciences, Farmingdale, NY, USA) were used as supplied.

SDS-PAGE reagents: acrylamide/bisacrylamide solution (37.5:1) at 40% (161-0148), TEMED (*N,N,N′,N′*-tetra-methyl-ethylenediamine, 161-0800) and APS (Ammonium Persulfate, 161-0700) were purchased to Bio-Rad Laboratories (Hercules, CA, USA). A broad range protein marker (New England BioLabs, Ipswich, USA, P7702S) was used in the electrophoresis assay. All reagents used were of analytical grade and all aqueous solutions were prepared in deionized water and stored at 4 °C.

2.2. Methods

2.2.1. On-chip protein kinase phosphorylation

Jerini phosphosite detector peptide arrays (Jerini Peptide Technologies, GmbH, Berlin, Germany) were used to identify potential phosphorylation sites within a library of forty-nine peptide sequences, derived from the endogenous proteins SLPI, Elafin, ECP and SP-D. Eight controls (Histone 1, 2, 3 and 4, myelin basic protein (MBP), alpha- and beta-casein and Tau protein) were also incorporated into the microarray format. The controls were spotted on the microarray in the four limits of each subarray (three subarrays per array – triplicates), serving simultaneously as positive controls and as landmarks for the identification of phosphorylation signals [32]. 13-Mers L-peptides were generated around *central* serine, threonine or tyrosine residues in order to improve accessibility to the phosphor-acceptor site. The phosphorylation on-chip was performed according to the PhosphoSiteDetector Protocol, supplied by the manufacturer. Briefly: peptide arrays were sealed with Gene-Frame^{TM} incubation chambers (AB 0630, Abegene, Epsom, UK), the chambers were filled with 330 μL of general kinase buffer (50 mM HEPES – NaOH, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 μM Na_3VO_4, 1 mM DTT and 1 μM ATP), casein kinase Iδ (10 units/mL, 8.56 μL) and ATP (1 mM, 33 μL). After 6 h of incubation at 35 °C, in a incubator shaker (Infors HT – Minitron, Bottmingen, Switzerland), the incubation chambers were removed from the slides and the microarrays were washed five times, for 5 min, with TBS buffer (50 mM Tris–HCl, pH 8.0, 137 mM NaCl and 2.7 mM KCl). Next, the chips were incubated with the blocking solution (blocking reagent and 100 mM maleic acid buffer, pH 7.5, 150 mM NaCl) for 1 h at 25 °C, rinsed with TBS buffer (five times for 5 min) and then dried with airflow. The use of blocking buffer after protein kinase incubation avoids unspecific binding of the phosphor-specific stain, which is applied in last step of this experimental procedure.

To detect the phosphopeptides on the microarrays (Fig. 1), a fluorescence phosphosensor dye – Pro-Q diamond stain (excitation/emission: 555/580 nm, respectively) was used. The microarrays were incubated with the stain for 60 min and then rinsed three times with destain solution, for a period of 30 min. This was followed by a final wash with ultra-pure water before drying in a nitrogen stream and analysing in a microarray scanner (Agilent and QuantArray).

2.2.2. Protein kinase phosphorylation in solution

The enzymatic activity of casein kinase Iδ (CKI) was measured using the PKLight^R HTS Protein Kinase assay Kit, from Cambrex (East Rutherford, NY, USA). The protein kinase activity measurements were performed following the manufacturer procedures. The kinase activity was measured in 96-well, white NUNC micro-plates using a total volume of 20 μL per well. Casein kinase Iδ was serially diluted in general kinase buffer, directly on the 96-well microplates, to give activities ranging from 3.75 to 1.83×10^{-3} units per well. Next, a mixture of substrate (8 μM Pep4 or Pep4M) and ATP (6 and 1 μM for Pep4 and Pep4M, respectively), diluted in general kinase buffer was added to all wells, including the negative controls (without casein kinase Iδ). The reaction mixture was incubated at 25 °C for 30 min, before adding 5 μL of kinase stop solution. The amount of ATP remaining was determined by adding 10 μL of ATP detection reagent to each well and incubating for 10 min at 25 °C (optimal temperature for the luciferase enzyme). The 96 well-plates were read at 560 nm on a BioTek, Synergy^{TM} HT (Winooski, VT, USA), in luminescence mode (Fig. 2).

2.2.3. Elastase activity measurements

The activity of porcine pancreatic elastase was assayed with the chromogenic substrate *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide [33], in accordance with the protocol supplied by the PPE manufacturer. The *p*-nitroanilide released was analysed in 1 mL quartz cells and monitored by recording the absorbance at 410 nm with a UV–vis Shimadzu spectrophotometer (UV-2501PC, Kyoto, Japan). Substrate concentration and enzyme activity of 4.4 mM and 0.3 units/mL, respectively, were used in the assay. The enzyme was pre-incubated at 25 °C with the inhibitor for 5 min before adding the substrate. The enzyme assays were conducted at 25 °C in 100 mM Tris–HCl buffer, pH 8.0. The hydrolysis rates of *N*-succinyl-$(Ala)_3$-*p*NA by PPE were acquired in the presence of inhibitors (Pep4, Pep4M) in concentrations ranging from 1 to 64 μM (Fig. 3). The non-peptide (non-hydrolysable) inhibitor elastatinal was used as a control and assayed in the same conditions as the peptides. An assay was also performed using the inhibitor-peptides concentrations that decreased mostly the elastase activity, both in the phosphorylated and non-phosphorylated