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Lung tissue destruction by proteinase 3 and cathepsin G mediated elastin degradation is elevated in chronic obstructive pulmonary disease

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterized by high levels of protease activity leading to degradation of elastin followed by loss of elasticity of the lung and the development of emphysema. Elastin is an essential structural component of the lung parenchyma to support the expansion and recoil of the alveoli during breathing. The lung extracellular matrix is vulnerable to pathological structural changes upon upregulation of serine proteases, including cathepsin G (CG) and proteinase 3 (PR3). In this study, we explored the diagnostic features of elastin neo-epitopes generated by CG and PR3. Two novel competitive enzyme-linked immunosorbent assays (ELISA) measuring CG and PR3 generated elastin fragments (EL-CG and ELP-3 respectively) were developed for assessment in serum. Both assays were technically robust and biologically validated in serum from patients with COPD. Serological levels of both elastin fragments were significantly elevated in patients with COPD compared to healthy controls. These data suggest that EL-CG and ELP-3 may serve as plausible biologic markers of destructive changes in COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by inflammation in the airways and an excessive concentration of neutrophils and mast cells [1,2]. Neutrophils and mast cells synthesizes and store serine proteinases like cathepsin G (CG) and proteinase 3 (PR3) in granules from which they are released in response to pro-inflammatory mediators. Together these proteinases have a broad spectrum of activity against the extracellular matrix (ECM) including the elastic fibers [3,4].

Elastic fibers are a major insoluble component of the ECM of the lungs. They are essential for structure, function, and elasticity of the lung tissue. The elastic fibers are comprised by an inner core of cross-linked elastin monomers, tropoelastin, embedded within fibrillin microfibrils [5,6]. They create a thin and highly branched network throughout the respiratory tree to support the expansion and recoil of the alveoli during breathing. They are characterized by a high stability, and a low turnover rate in healthy adult tissue, with a half life estimated to 40 years [7,8]. Only a few proteases such as serine proteinases in addition to selected matrix metalloproteinases (MMPs) are able to cleave elastic fibers. These proteinases are known as elastases [9–11]. In a normal inflammatory response the protease-protease inhibitor balance is maintained through the secretion of endogenous protease inhibitors e.g. heparan sulfate [9], tissue inhibitors of metalloproteinases (TIMPs) [12] or α_1-Antitrypsin [13]. In pathological conditions this balance is skewed which can lead to a loss of elastic fibers which is a major pathological feature of COPD and emphysema [14,15]. Several studies suggest that both the serine proteinases CG and PR3 are up regulated in COPD, but there are currently no easy way to link increased degradation of elastin with the two elastases. In this study we aimed to investigate if CG and/or PR3 degradation of elastin is increased in COPD assessed serologically. For that purpose we established and evaluated two competitive ELISAs specific for

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Abbreviations

BMI	Body mass index
CG	Cathepsin G
COPD	Chronic obstructive pulmonary disease
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assays
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
h	hours
HNE	Human neutrophil elastase
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
min	minutes
MMPs	matrix metalloproteinases
PR3	Proteinase 3
RE%	Recovery percentage
TBS	Tris-buffered saline
TIMPS	Tissue inhibitors of metalloproteinases
ULOD	Upper limit of detection

elastin fragments generated specifically by CG and PR3, respectively.

2. Methods

2.1. Reagents

All applied reagents used in the presented experiments were high quality chemicals from Sigma Aldrich (St. Louis, MO, USA) and Merck (Whitehouse Station, NJ, USA). The 96-well streptavidin-coated microtiterplates used were from Roche, Basel, Switzerland. The assay buffer applied consisted of 50 mM Tris-buffered saline (TBS) 2 g NaCl, pH 8.0. 3,3′,5,5' –Tetramethylbenzidine (TMB) was from Kem-EN-Tec Diagnostics (Taastrup, Denmark). The synthetic peptides used for immunization and assay development were 1) Immunogenic peptides: KLH-CGG-LGGVAARPGF (EL-CG) and KLH-CGG- LPGGYGLPYT (ELP-3), 2) Biotinylated peptides: LGGVAARPGF-Biotin (EL-CG) and Biotin- LPGGYGLPYT (ELP-3), 3) Standard peptides: LGGVAARPGF (EL-CG) and LPGGYGLPYT (ELP-3) and 4) Elongated peptides: LGGVAARPGFG (EL-CG) and LPGGYGLPYTT (ELP-3), 5) Truncated peptides: LGGVAARPG (EL-CG) and LPGGYGLPY (ELP-3), 6) Nonsense peptide: PGGVPGGVFY (for both assays) and 7) Nonsense biotinylated peptide: PGGVPGGVFY-K-biotin (for both assays). All synthetic peptides were purchased from American peptide Company, Sunnyvale, California USA. Reagents applied for *in vitro* cleavages: Elastin (Sigma, E7152), human neutrophil elastase (HNE) protein (Abcam, ab91099), PR3 (EPC ML734), CG (EPCSG623) and complete protease inhibitor (Roche 1186153001).

2.2. Selection of target sequence

Cleavage sites specific for CG and PR3 in elastin (Uniprot: P15502 – ELN_Human) have previously been identified by mass spectrometry [16]. Based on these unique CG and PR3 generated cleavages of elastin decapeptides were blasted for homology to decapeptide sequences from other proteins using the NPS@: network protein sequence analysis. From this two sequences unique for elastin were selected: the sequence LGGVAARPGF (specific for CG cleavage) was selected for the EL-CG assay and LPGGYGLPYT (specific for PR3 cleavage) selected for the ELP-3 assay antibody development (Table 1).

2.3. Antibody development

Immunizations were performed in 6 mice, for each selected target sequence. The mice were 4–6 weeks old and immunized with 200 μl emulsified antigen (100 μg per immunization (KLH-CGG-LGGVAARPGF (EL-CG) and KLH-CGG- LPGGYGLPYT (ELP-3))) mixed with Sigma adjuvant subcutaneously in the abdomen. The immunizations were performed every second week. The mice (one for each sequence) with the highest serum immune response were selected for fusion. The selected mice were rested for a month and then boosted intravenously with 100 μg immunogenic peptide in 100 μl 0.9% NaCl solution three days before isolation of the spleen. The spleen cells were fused with SP2/o myeloma cells to produce hybridroma as described by Gefter et al. and cloned in culture dishes [17]. The clones were plated into 96-well microtiter plates employing the limited dilution method to ensure monoclonal growth. Supernatants of antibody-producing hybridoma were screened for specificity against standard peptide and native material in an indirect ELISA using streptavidin-coated plates. The biotinylated peptides LGGVAARPGF-Biotin (EL-CG) and Biotin-LPGGYGLPYT (ELP-3) were used for screening, while standard peptides LGGVAARPGF (EL-CG) and LPGGYGLPYT (ELP-3) were used as calibrators to test for further specificity of clones.

2.4. The EL-CG and ELP-3 ELISA establishment and protocol

Supernatant from two selected clones for each of the targeted sequences were collected and monoclonal antibody was purified using HiTrap affinity columns (GE Healthcare Life Science, Little Chalfront, Buckinghamshire, UK).

Using the purified antibodies, the EL-CG and ELP-3 assays were developed as competitive ELISA assays. Different buffers and incubation times were tested leading to the following protocol: For each assay a 96-well streptavidin-coated microtiter plate (Roche, Basel, Switzerland) was coated with biotinylated peptide (100 μl) dissolved in assay buffer for 30 min at 20 °C with shaking. Plates were washed five times in washing buffer (20 mM TRIS, 50 mM NaCl, pH 7). Sample/standard peptide/control (20 μl) was added and followed by addition of monoclonal antibody (100 μl) and incubated for 3 h at 4 °C with shaking. After incubation, plates were washed five times in washing buffer. Secondary HRP-labeled goat anti-mouse antibody was added (100 μl) followed by 1 h incubation at 20 °C with shaking. Plates were than washed and TMB (100 μl) was added for 15 min, the reaction was stopped by 0.1 M sulfuric acid (100 μl). The plate was then analyzed by a SpectraMax M reader (Molecular Devices, Ca, USA) at 450 nm with 650 nm as the reference. A standard curve was plotted using a 4-parametric mathematical fit model. Each ELISA plate included kit controls to monitor inter- and intra-assay variation.

Table 1
The selected sequences of the two assays. The antibodies recognizes the neo-epitopes originating from amino acid residues 213–222 (ELP-3) and 756–765 (EL-CG) in the human protein.

Assay	Cleavage site	Protease	Peptide sequence
EL-CG	c-terminal (765–766)	Cathepsin G	756′-LGGVAARPGF-765
ELP-3	c-terminal (222–223)	Proteinase 3	213′-LPGGYGLPYT-'222

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