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Comparison of the properties of rare variants of α_1-proteinase inhibitor expressed in COS-1 cells and assessment of their potential as risk factors in human disease

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Abstract

Among the more than 75 known variants of α_1-proteinase inhibitor, a sub-population of rare, point mutations causing single amino acid replacements have been identified and classified as "at risk" alleles for development of pulmonary disease. In most cases, it is not clear how the amino acid replacements typical of these variants change the properties of the inhibitor to increase risk of disease in the affected individuals. To begin to address this question, we mutagenized a wild type α_1-proteinase inhibitor cDNA to encode a panel of eight different point mutants reported to be associated with increased risk for development of pulmonary disease. These variants were then expressed in COS-l cells transiently transfected with plasmids containing the altered cDNAs. The effects of the mutations on the rates of secretion, cellular location, intracellular degradation, activity, stability, and tendency to aggregate were determined. Results of these studies show that, in some cases, the mutations affect the rate of secretion, the activity or both of these properties of α_1-proteinase inhibitor in a manner consistent with its designation as an "at-risk" allele. In other cases, the mutations do not significantly change the properties of the inhibitor, suggesting that these may be normal variants and that their expression may not increase the risk of disease.

Keywords: α_1-proteinase inhibitor; Point mutant; Elastase; Binding-activity; Secretion; Stability

1. Introduction

Human alpha l-proteinase inhibitor (α_1Pi, α_1-antitrypsin)[1], a member of the serpin super family of proteins, is the major serine protease inhibitor in the circulation and functions primarily to inhibit elastase released from activated or disintegrating neutrophils [1]. A deficiency of circulating α_1Pi is associated with pulmonary emphysema and childhood liver disease [2,3]. The pulmonary damage appears to be a direct result of the failure to adequately control the activity of the neutrophil elastase either due to too little circulating inhibitor, to the lack of activity of the secreted α_1Pi, or to a combination of both. While a number of variants of α_1Pi have been described, most individuals express the normal M allele [4] and have circulating levels of α_1Pi in the range of 104 to 276 mg/dl [3]. Serum levels of active α_1Pi above 57 mg/dl are adequate to protect the lung from elastase digestion [3]. If serum levels of α_1Pi fall below this threshold or if the circulating inhibitor is an inefficient inhibitor of elastase, lung damage leading to emphysema can result.

The Z allele is most commonly associated with a deficiency state severe enough to cause disease [3]. Individuals homozygous for this allele or who express it in combination with other deficiency alleles, are at risk of developing emphysema and/or liver disease. These individuals appear to produce normal amounts of α_1PiZ to transport

Abbreviations: α_1-proteinase inhibitor, α_1Pi; Endoplasmic reticulum, ER; Phenylmethylsufonyl fluoride, PMSF; Transverse urea gradient, TUG

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the protein into the lumen of the endoplasmic reticulum (ER), but are unable to efficiently transport the protein from the ER to the Golgi apparatus [5–7]. The inefficiently secreted α_1PiZ is largely degraded, probably by a mechanism involving ubiquitination, retro-translocation into the cytosol, and subsequent degradation by the proteasome [8–10]. Smaller amounts of α_1PiZ accumulate as inclusion bodies in cells expressing this variant [11]. Accumulation of α_1PiZ and formation of inclusion bodies is thought to lead to the liver damage occurring in about 15% of the Z homozygotes [12]. The basis for the liver damage is not well understood but expression of α_1PiZ in skin fibroblasts isolated from the different populations of Z homozygotes indicate that those who develop liver disease are less able to degrade the retained α_1PiZ [13]. As a result of the inefficient secretion of the Z variant, the circulating levels of this form of the inhibitor are well below the 57 mg/dl threshold needed to protect the lungs. The severity of the deficiency state is probably magnified due to a reduction in the ability of α_1PiZ to inhibit elastase [14]. Both the inefficient secretion and the reduction in the activity of the inhibitor result from a point mutation leading to the replacement of Glu^{342} with Lys [6,7,15]. The means by which this mutation causes the altered properties of α_1Pi are not known.

While the Z allele is found at a frequency of about 0.01 to 0.02 in many populations [2], other rarer variants of α_1Pi harboring single amino acid replacements have been reported to be associated with pulmonary disease. None of these rare variants has been as thoroughly studied as α_1PiZ and the consequences of these mutations upon the properties of α_1Pi are largely unknown. This study was undertaken to systematically compare the rates of secretion, the stabilities, the sites of accumulation, and the activities of eight of these rare variants after their expression in Cos cells. The variants studied are: α_1PiI, Arg^{39}→Cys [16]; $\alpha_1PiM_{procida}$, Leu^{41}→Pro [17]; α_1PiS_{iiyama}, Ser^{53}→Phe [18]; $\alpha_1PiM_{mineral\ springs}$, Gly^{67}→Glu [19]; $\alpha_1PiQO_{ludwigshafen}$, Ile^{92}→Asn [20]; α_1PiP_{lowell}, Asp^{256}→Val [21]; $\alpha_1PiW_{bethesda}$, Ala^{336}→Thr [22]; and $\alpha_1PiM_{heerlen}$, Pro^{369}→Leu [23].

2. Materials and methods

2.1. Reagents

Porcine pancreatic elastase (type IV) and human leukocyte elastase were purchased from Sigma Chemical Co. (St. Louis, MO). Kits for mini preparation of plasmid DNA and for extraction of DNA fragments from agarose gels were obtained from Qiagen Inc. (Chatsworth, CA). Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase and Hams F-12 growth medium, complete and methionine free, were provided by GibcoBRL (Grand Island, NY). $Vent_R$ DNA polymerase was purchased from New England BioLabs, Inc. (Beverly, MA). Fluorescein isothiocyanate-goat anti-rabbit IgG was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Oligonucleotides used as primers for PCR and for DNA sequencing were synthesized by Molecular Resources (Fort Collins, CO). [^{35}S]-L-methionine, [a-^{35}S]-deoxyadenosine-5′-triphosphate, and Na ^{125}I were purchased from New England Nuclear (Boston, MA).

2.2. Site directed mutagenesis

The production of all of the variants except $\alpha_1PiQO_{ludswigsbafen}$ and $\alpha_1PiW_{bedmsda}$ was accomplished by use of the recombinant polymerase chain reaction (RPCR) [24] to change the appropriate nucleotide in pSV7α_1PiM to yield the necessary amino acid change. The polymerase chain reactions for each mutation were carried out at three different concentrations, 1, 10, and 100 ng, of alkali denatured pSV7α_1PiM [25]. The other components of the reaction mixtures were 0.8 μM of the appropriate primers (Table 1), 0.2 mM of all four deoxynucleotide triphosphates, 2 units of $Vent_R$ polymerase, 10 mM KC1, 20 mM Tris–HCI (pH 8.8 at 25 °C), 10 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$ in a final volume of 100 μl. The reactions were carried out in a BioOven thermocycler (BioTherm Corporation, Arlington, VA) programmed for cycles of 1 min denaturation at 94 °C, 50 s annealing at 60 °C; and 5 min elongation at 72 °C. At the end of 30 cycles, the reactions were maintained at 72 °C for 10 min to encourage completion of full-length product. The products were linear, blunt-ended, double-stranded DNAs with identical overlapping ends that contained the complete plasmid sequence (3679 to 3682 nucleotides, depending upon the primer set used). The linear DNA fragments were purified by electrophoresis through 1% agarose gels, excising gel slices containing the appropriately sized fragment, and extracting the DNA using a DNA gel extraction kit. One to 10 ng of the purified, linear DNA was introduced into electrocompetent E. coli DH5-a (40 μl of a suspension of 2×10^{10} cells/ml in a chilled 0.1 cm cuvette) by electroporation using a Bio Rad-Gene Pulser™ (BioRad, Hercules, CA) with setting of 25μF, 1.65 kV, and 200 Ω. The electroporated cells were incubated in SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCI, 10 MM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at 37 °C for 1 h, plated onto ampicillin selective medium and incubated over night at 37 °C. Three milliliter aliquots of L broth were inoculated with cells from 4 to 6 of the resulting colonies and incubated overnight at 37 °C. Plasmid DNA was prepared from these cultures by standard procedures [26] and purified using a DNA purification kit following the manufacturer's directions. The regions of the plasmids that potentially contained the desired mutation were then sequenced using appropriate primers and the TaqTrack Deaza Sequencing System according to the manufacturer's directions (Promega Corp., Madison, WI). Those found to have the necessary base changes were selected to complete construction of the vectors.