Qualitative Study of Fecal α1-Proteinase Inhibitor in Normal Subjects and Patients with Crohn's Disease

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We applied sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting to analyze fecal α1-proteinase inhibitor (α1PI) from healthy subjects and patients with Crohn’s disease. A component with M, 38 000 was characterized in normal fecal extracts as well as in six pathological samples. In these cases, the Crohn’s disease activity index (CDAI), a clinical index of severity of the disease, was 170 (SEM 47). In contrast, α1PI of M, 51 000 was detected in fecal extracts from eight patients with active Crohn’s disease (CDAI = 287, SEM 39). We conclude that fecal α1PI can be considered a marker of intestinal disease activity.

Additional Keyphrases: α1-antitrypsin • immunoblotting • sodium dodecyl sulfate–polyacrylamide gel electrophoresis • intestinal disease

Protein losses are commonly encountered in inflammatory bowel disease. Protein-loss enteropathy under these circumstances has been assessed by measuring fecal excretion of 51Cr-labeled albumin and more recently, of α1-proteinase inhibitor (α1PI) (1). Fecal clearance of α1PI has been shown to be well correlated with excretion of 51Cr-labeled albumin; however, its use as a marker of inflammatory bowel disease activity remains poorly established (2)—possibly because of biochemical changes of α1PI in the intestine of such patients, resulting in an apparent decrease of the fecal concentration of α1PI as it is usually measured (radial immunodiffusion). Indeed, recent reports have mentioned fecal complexes of α1PI with proteases in patients with ulcerative colitis (3) and necrotizing enterocolitis (4).

Therefore, the aim of this study was to determine the biochemical characteristics of fecal α1PI in patients with Crohn’s disease as compared with normal volunteers.

Materials and Methods

Subjects. We selected for this study 16 patients with Crohn’s disease, having different involvement and (or) location (five males, 11 females; median age: 27 y, range 15–64). Six of the patients had ileitis, eight had ileocolitis, and two had pure colitis. The mean duration of disease was 4.8 y (range 0.16–20).

The activity of the disease was monitored by calculation of a Crohn’s disease activity index (CDAI) (5). We used one based on clinical variables: diarrhea, abdominal pain, general well-being, presence of an abdominal mass, body weight loss, and intestinal or systemic complications.

We also determined fecal α1PI in a group of 10 healthy controls (six males, four females; median age: 30 y, range 15–65 y).

Reagents. Anti-α1PI immunoglobulins were provided by Dako (Sèbia, Issy-les-Moulineaux, France); porcine pancreatic elastase (EC 3.4.21.36) by Choay, Paris, France; bovine serum albumin and fetal calf serum by Boehringer Meylan, France. Nitrocellulose sheets (0.45-μm pore size) were purchased from Schleicher & Schuell (Ceralabo, Aubervillers, France). Acrylamide and N,N'-methylenebisacrylamide were provided by BDH Ltd., Poole, U.K. All chemicals used were of analytical grade.

Pure native α1PI was isolated according to Sugiyama et al. (6). α1PI-porcine pancreatic elastase complex and degraded α1PI were prepared by incubating α1PI and porcine pancreatic elastase at a molar ratio (inhibitor/enzyme) of 1/2, in phosphate-buffered saline (phosphate 10 mmol/L, NaCl 150 mmol/L, pH 7.5). After 30 min of incubation at room temperature, we stopped the action of elastase by adding a 100-fold molar excess of phenylmethylsulfonylfluoride (PMSF) with respect to the protease initially present. The resulting solution was immediately frozen (at −20 °C) in 0.5-mL aliquots for subsequent comparisons.

Preparation of stools. Twenty-four-hour stools, collected for three consecutive days, were weighed and immediately frozen at −20 °C. After mixing the whole sample from each subject (all three days), we removed 2 g of the homogenized material, vigorously shook this for 10 min with 4 mL of 0.15 mol/L NaCl solution, and centrifugated (1000 × g, 10 min) it. The supernatant liquid was separated and mixed with a solution of PMSF (final concentration 1 mmol/L) in isopropanol. We determined the α1PI concentration in this supernate, assuming a threefold dilution of the stool sample.

Fecal α1PI determination. We measured fecal α1PI by single immunodiffusion, using Nor-Partigen plates (Behring-Diagnostik, Rueil-Malmaison, France). Ring diameters were read after 72 h and the concentrations calculated by comparison with a control plasma (Behring Diagnostik). When the α1PI content was low, we used LC-Partigen plates and calculated the concentration from a reference curve prepared from a commercial serum standard (LC-V, Behringwerke). Fecal α1PI was expressed as milligrams per gram of stool (wwg weight).

Electrophoresis procedures. For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) we used 10% polyacrylamide slab gels, at pH 8.3, with the Laemmlı (7) buffer system and a Protein II chamber from BioRad (Touzart et Matignon, Vitry-s-Seine, France). Each sample, containing about 5 μg of α1PI, was reduced by mercaptethanol before loading twice on the gel. Two different stainings were performed on each sample: after migration,
half of the gel was fixed with picrate then stained overnight with Coomassie Brilliant Blue (8). Proteins loaded on the remaining part of the gel were transferred onto nitrocellulose sheets as described by Towbin et al. (9), at 12 V/cm in a Transblot cell (BioRad). Immunological detection was performed according to Hawkes (10).

Crossed immunoelectrophoresis was carried out according to Clarke and Freeman (11), with use of anti-α1PI immunoglobulins (Dako).

Statistical analyses. We used Student's t-test for unpaired data.

Results

Incubation of human α1PI (Mr 54 000) with an excess of porcine pancreatic elastase formed a protease-antiproteinase complex having an apparent Mr of ~79 000 in SDS-PAGE. A modified form, formed by partial hydrolysis of the previous complex (Mr about 51 000), was also characterized, as previously described by Satoh et al. (12). These derivatives were well separated by electrophoresis in SDS-PAGE (Figure 1A, lanes a and b) and were detected immunologically with specific anti-α1PI immunoglobulins, after being transferred onto nitrocellulose (Figure 1B, lanes a and b). We used these derivatives as reference points.

When analyzed under the same conditions, the fecal extracts from control subjects always exhibited an α1PI form with an apparent Mr of 38 000, which was also present in six samples from patients with Crohn's disease (Figure 1B, lane 2). However, in eight cases of Crohn's disease, α1PI exhibited a Mr of 51 000 (lanes 1, 3, 4, 5); in two cases, it migrated with an intermediate mobility, near Mr 45 000 (lanes 5, 7). In the samples studied, α1PI was generally characterized as a single band.

In all cases, the mobility of α1PI characterized in fecal extracts was not related to the amount of α1PI excreted per 24 h, as measured on three days (Figure 2): 275 ± 127 mg/24 h and 436 ± 160 mg/24 h (mean ± SEM, differences not significant) for α1PI of Mr 38 000 and 51 000, respectively.

No relationship was established between the form of fecal α1PI excreted in stools and the sex or age of the patient, the duration of the disease, or its location.

Six patients had α1PI (Mr 38 000) associated with a mean CDAI = 170 (SEM 47). The α1PI (Mr 51 000) was characterized in stools from eight patients with a CDAI = 287 (SEM 39), significantly different index values (P <0.05). α1PI (Mr 51 000) was thus more frequently associated with an increased activity of the disease as indicated by CDAI (Figure 3).

Under our conditions, immunoblot analysis of contrived specimens clearly detected ~0.5 μg of α1PI complexed with elastase (i.e., 10% of α1PI loaded on the gel). In the subjects' samples we studied, such complexes (apparent Mr ~79 000) were not detected.

In crossed immunoelectrophoresis, complexes of α1PI with elastase are detected as a cathodal peak (4). No such peaks were visible in stool supernates containing either Mr 38 000 or 51 000 form of α1PI. The fecal form of α1PI was present as
a single prominent peak, with electrophoretic mobility near that of serum a1-PI. Nevertheless, in some samples containing a1-PI (M, 51 000), an additional small peak was detected that had an intermediate mobility between degraded a1-PI and a1-PI-elastase complex.

After staining of the polyacrylamide gels with Coomassie Brilliant Blue, a band with an identical mobility to a1-PI, detected immunologically, was sometimes clearly visible in stool samples (for comparison, see lane 2 in Figure 1A and 1B). Additional bands were also stained. In patients’ samples, these bands generally migrated farther than serum albumin.

**Discussion**

In this study, we demonstrated the presence of a modified form of a1-PI (M, 38 000) in fecal extracts from healthy subjects as well as from some patients with Crohn’s disease. Some of the patients were in remission (CDAI ≤150). Therefore, we assume that a1-PI undergoes a pronounced degradation during its intestinal transit by pancreatic and bacterial proteolytic enzymes normally present. The cleaved form of a1-PI so characterized may be similar to the low-M, species of a1-PI (M, 34 000) previously described by Travis et al. (13), which is the post-complex product of the in vitro interaction between native a1-PI and human chymotrypsin-like enzymes.

On the other hand, a1-PI (M, 51 000) was characterized in some fecal extracts from patients with Crohn’s disease. This form was always associated with an increased severity of the disease, as indicated by CDAI. From these observations, we speculate that in these patients native a1-PI combines with leukocyte elastase in the inflamed intestinal mucosa. Granulocytic elastase–a1-PI complexes have been clearly characterized in feces from patients with acute colitis (3); likewise, such complexes have been found in increased concentrations in plasma from patients with active Crohn’s disease (14). The further dissociation of such complexes generates an inactive form of a1-PI (M, 51 000) by cleavage of its reactive site 389–390 activity (15). This form is strongly resistant to further proteolytic degradation (16) and therefore could be demonstrated at a higher concentration in fecal extracts from patients with an active inflammatory bowel disease, as assessed by CDAI.

We could not characterize complexes of proteases–a1-PI by immunoblot analysis. These complexes were present only in minor amounts in some stools from patients with acute ulcerative colitis (17). In contrast, they are commonly found in newborns at risk for necrotizing enterocolitis (4); in this case, the shortness of transit could be consistent with the limited stability of such complexes.

Previous investigations have shown that denaturing native a1-PI makes it much more labile and more susceptible to proteolysis (16). Indeed, physiological concentrations of bile salts induce conformational changes, with inactivation of a1-PI (18). We hypothesize that native a1-PI, which leaks into the intestinal lumen, is in part degraded into small peptides that do not present any immunological reactivity. This would explain the underestimation of enteric protein loss, as measured by a1-PI clearance determination (2).

Furthermore, native, complexed, and partly proteolyzed forms of a1-PI all react differently in immunological assays (19). Effectively, we have isolated a1-PI of M, 38 000. When evaluated by radial immunodiffusion with a commercial standard (Behring) as calibrator, its apparent concentration might be underestimated (by ~20%) when compared with native a1-PI (preliminary results). Further isolation of other forms of a1-PI in feces might establish their relative immunoreactivity.

Our findings are consistent with characterization of a particular form of a1-PI (M, 51 000) in stools from patients with active Crohn’s disease. Qualitative assays of fecal a1-PI need further examination in a prospective study of a large group of patients with exudative enteropathies, especially Crohn’s disease, to determine the potential usefulness of this protein as a marker of intestinal disease activity.

We thank Marie-Dominique Romain for her excellent technical assistance, which is gratefully appreciated.

**References**