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Polypeptide marker and disease patterns found while mapping proteins in ascitis

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ABSTRACT

To assess the protein composition of ascitis, 28 samples of ascitic fluid were obtained from patients admitted to Geneva University Hospital. The samples were analysed randomly and blindly by high-resolution two-dimensional polyacrylamide gel electrophoresis. The final visual evaluation was compared with the discharged summary and diagnosis. The protein pattern of ascitis was, as expected, very similar to normal or diseased plasma, with the exception of two spots which were present in ascitic fluids but not in the 200 plasma samples analyzed in parallel. After microsequencing, they proved to be β-fibrinogen fragments. Several diseases showed distinct patterns, especially acute pancreatitis. A group of intense spots with an apparent molecular mass between those of α_1-antitrypsin and β-haptoglobin were found in all ascitic fluid from pancreatitis cases (six patients). These spots had isoelectric points similar to those of α_1-antitrypsin and β-haptoglobin and microsequencing revealed that they were three different fragments of α_1-antitrypsin.

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INTRODUCTION

Physiopathologically and schematically, the accumulation of ascitic fluid is due either to increased capillary pressure, to increased capillary permeability or to decreased blood oncotic pressure. Lymphatic obstruction can also lead to ascitis formation. There are several causes of the accumulation of ascitis. In the western world, the most common diseases associated with ascitis are ethanol-induced liver cirrhosis, cardiac insufficiency, neoplasia and, less frequently, tuberculosis.

Often, patients' history and physical examination provide enough information to determine the etiology of ascitis. Sometimes, laboratory tests, cytology and culture are required in order to make the final diagnosis. Among the laboratory tests, the measurement of the protein concentration helps to determine whether the fluid is a transudate (protein concentration <25 g/l as in most cardiac insufficiencies and alcoholic liver cirrhosis) or an exudate (protein concentration >25 g/l as in pancreatitis or peritoneal carcinosis). Some protein measurements, such as lactate dehydrogenase, amylase or lipase, also give valuable information to make a diagnosis.

To evaluate more thoroughly the protein content of ascitis and to look for specific protein markers, samples of ascitic fluid from patients admitted to the Medicine, Surgical, Obstetrics and Gynecology Departments of Geneva University Hospital were obtained and analysed by high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and were compared with normal and diseased plasma maps.

EXPERIMENTAL

Reagents

Acrylamide, piperazinediacrylamide, tetramethylethylenediamine, ammonium peroxodisulphate, glycine, sodium dodecyl sulphate (SDS) and poly(vinylidene difluoride) (PVDF) membranes were obtained form Bio-Rad (Glattbrugg, Switzerland), tris(hydroxymethyl)aminomethane (Tris) hydrochloride, Nonidet P-40 and cholamidopropyl dimethylhydroxypropanesulphonate from Sigma (Deisenhofen, Germany), citric acid, urea and dithiothreitol from Merck (Darmstadt, Germany) and Resolytes 4–8 from Brunschwig (Basle, Switzerland).

Apparatus

Unless stated otherwise, all the electrophoretic equipment used was from Bio-Rad. The isoelectrophoretic separation was performed in a Model 175 chamber with a Model 3000/300xi power supply. Preparative isoelectrofocusing was done using a Rotofor apparatus. For SDS-PAGE, a Protean II chamber and casting chamber (160 × 200 × 1.5 mm gels) were used. The power supply (700 V, 1.6 A) was from Hewlett-Packard (Palo Alto, CA, USA). The gradient pourer was a Bio-Rad Model 395 and, more recently, an Angelique computerized gradient pourer (Large Scale Biology, Rockville, MD, USA). N-Terminal sequence determinations were performed using a Model 473A microsequencer (Applied Biosystems, Foster City, CA, USA) equipped with a Problott reaction cartridge.

Sample collection and preparation

Blood samples were collected in EDTA tubes and immediately centrifuged at 2000 *g* for 5 min at 5°C. Plasma aliquots were made and stored frozen at −20°C. Ascitic fluids were collected in sterile tubes either in the operating room, in the emergency room or on the ward, by abdominal tap with a sterile 22-gauge needle and a 20-cm^3 syringe. They were centrifuged to remove any fragments or floating cells, then aliquots were made and stored frozen. The addition of proteinase inhibitors, when collecting the sample, did not modify the protein pattern.

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was performed essentially, as described by Hochstrasser and co-workers [1,2]. For analytical purposes, 0.3 μl of plasma and 0.6 μl of ascitic fluid were separated in the first dimension. After the second dimension, the gels were stained with silver [2,3]. When 2D-PAGE

was followed by electroblotting and microsequencing analysis (see below), the amount of sample loaded in the first dimension was multiplied by 33. In some experiments, 25 ml of ascitic fluid were first separated by preparative isoelectrofocusing in a Rotofor chamber as described previously [4]. Then, 40 μl of each fraction were separated by 2D-PAGE and silver-stained to localize the fraction of interest. The appropriate fraction was lyophilized and redissolved in the smallest volume of distilled water to solubilize urea and electrolytes. In general, half the entire fraction was separated by 2D-PAGE and electroblotted.

Electroblotting and staining procedures

Electroblotting onto PVDF membranes was done essentially according to Towbin *et al.* [5] and Matsudaira [6]. Membranes were stained with amido black and destained with water. Spots of interest were excised, dried under nitrogen and kept in Eppendorf tubes at −20°C until microsequencing was performed.

RESULTS AND DISCUSSION

Ascitic fluid does not exist in a normal situation. Consequently, and because of the great similarity, ascitis protein maps were compared with plasma maps. Most of the 300–700 spots found in the ascitis protein map were also detected in the plasma map (Fig. 1).

Two spots, which were never detected on more than 200 normal and diseased plasma maps that

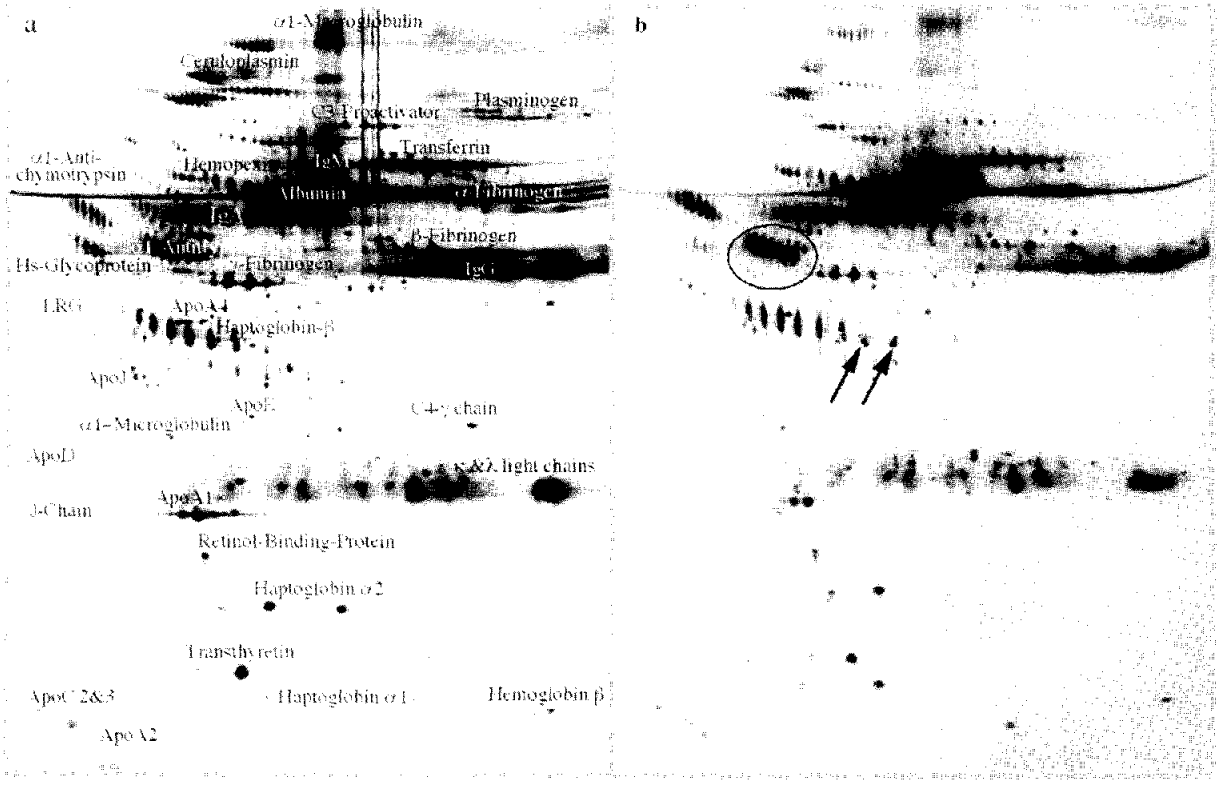

Fig. 1. (a) Typical normal plasma protein map. A 0.3-μl sample of plasma (or about 18 μg of protein) was loaded on this 2D-PAGE gel. Proteins were detected after silver staining. (b) Map of ascitic fluid. A 0.6-μl aliquot of ascitic fluid (or about 18 μg of protein) was loaded on the gel. The proteins were identified either by comparison with Anderson *et al.* [8], by the immunoblotting technique [5] or by N-terminal microsequencing [6]. The arrows highlight two spots typical of ascitic fluid (see Fig. 2). The oval highlights the area of interest in acute pancreatitis (see Fig. 3).

TABLE I

N-TERMINAL SEQUENCES OF SOME ASCITIC FLUID POLYPEPTIDES

This table highlights the N-terminal amino acid sequence of eight ascitic proteins which were blotted onto PVDF membranes. Two spots, not seen on plasma map but present in all ascitis maps, were β-fibrinogen fragments. A group of spots found in acute pancreatitis were identified as α_1-antitrypsin fragments.

Group of spots No. in	N-Terminal sequence	Position in:	Present in normal plasma	Present in ascitis	Present in ascitic fluid in case of pancreatitis
Fig. 2		β-Fibrinogen precursor			
a	Asp-Asn-Glu-Asn-Val-Val-Asn-Glu	164	No	Yes	Yes
b	Asp-Asn-Glu-Asn-Val-Val-Asn-Glu-	164	No	Yes	Yes
Fig. 3		α_1-Antitrypsin			
1	Glu-Asp-Pro-Gln-Gly-Asp	1	Yes	Yes	Yes
2	Asp-Ala-Ala-Gln-Lys-Thr	6	Yes	Yes	Yes
3	Glu-Asp-Pro-Gln-Gly-Asp-Ala-Ala-Gln-Lys-Thr-Asp-Thr	1	Yes	Yes	Yes
4	Glu-Asp-Pro-Gln-Gly-Asp-Ala-Ala	1	No	No	Yes
5	Asp-Ala-Ala-Gln-Lys-Thr	6	No	No	Yes
6	Asp-Gln-Asp-His-Pro-Thr-Phe-Asn	17	No	No	Yes

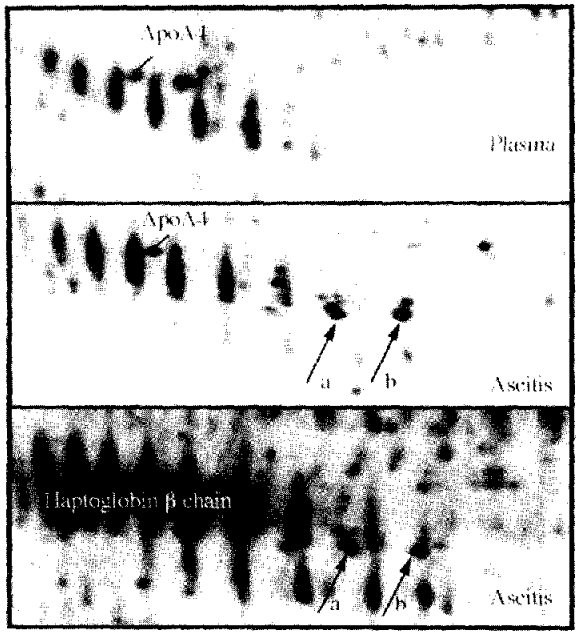

Fig. 2. Illustration of the two spots (a) and (b) found in all ascitic fluids but never in plasma. The three blow-up pictures show the area of the haptoglobin β-chain and Apo A4 lipoprotein. The N-terminal microsequence revealed that spots a and b are both fragments of the β-profibrinogen molecule (see Table I).

we have analysed, were seen on every ascitis map in the region of the haptoglobin β-chain (Fig. 2). After 2D-PAGE and electroblotting followed by microsequencing, they were found to be fragments derived from fibrinogen β-chain. The N-terminal sequences of both fragments were identical and correspond to position 164 of the fibrinogen β-chain precursor (Table I). Surprisingly, the same two spots were identified in the spinal fluid of schizophrenic patients and were thought to be specific to that disorder [7].

Between α_1-antitrypsin and haptoglobin β-chain, a group of additional spots were detected in much higher concentration in all six pancreatitis cases than in the remaining 22 patients or than in the plasma maps of the same patients (Fig. 3). Their partial microsequence and apparent molecular mass provided their identification as α_1-antitrypsin fragments. As detailed in Table I, their N-terminal sequences correspond to positions 1, 6 and 17 of the native α_1-antitrypsin molecule. Similar modifications, but with a different glycosylation pattern, were seen in inflamed joint fluid and bone cyst maps. The presence of these additional spots could be explained by the increased enzymatic activity associated with these diseases. Indeed, in severe pancreatitis, proteolytic enzymes can be released in the abdomen, where they induce severe abdominal necrosis. The pat-

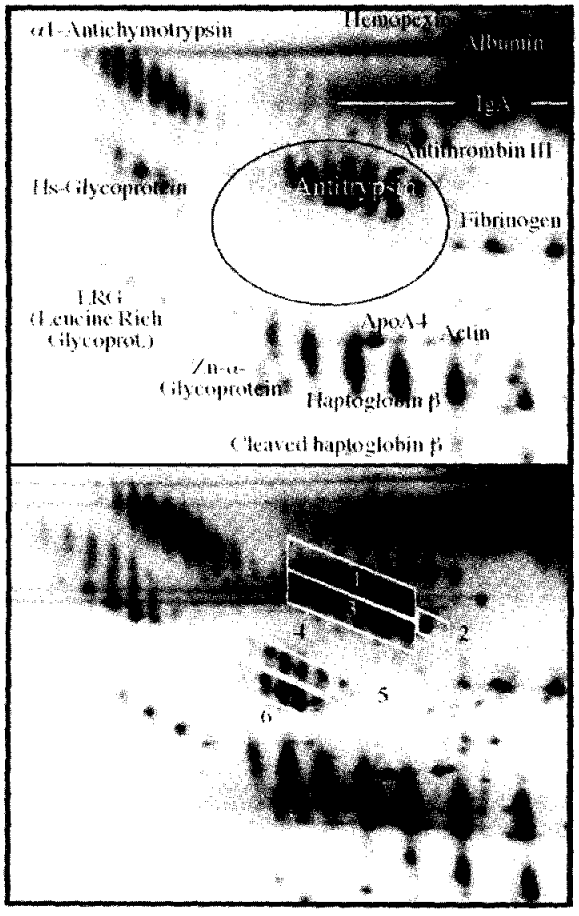

Fig. 3. The top picture shows the α_1-antitrypsin and derived fragments region of ascitic fluid in cirrhosis. The bottom picture shows the same area but in acute pancreatitis. The groups of spots numbered 1–6 were microsequenced. The results are shown in Table I. All spots are α_1-antitrypsin or α_1-antitrypsin fragments. The appearance of α_1-antitrypsin fragments (spots 4–6) in acute pancreatitis is certainly due to the release of protease(s) by the pancreas and the digestion of α_1-antitrypsin at its cleavage site. The pattern of spots is due to the glycosylation heterogeneity, each additional sugar molecule increasing the apparent molecular mass and adding more negative charges to the polypeptide chain.

tern of α_1-antichymotrypsin seemed to follow the same pattern modification. However, so far, the magnitude of the changes does not seem to correlate with the disease gravity and prognosis.

The immunoglobulin pattern correlated well with the disease diagnosis and severity. Massive IgA increases were seen in the ten cases with liver cirrhosis; IgM and IgG increased in acute and chronic infections with a polyclonal stimulation seen in the light chain area in five out of seven patients. In malignancies, although no specific changes were found, the amount of immunoglobulins generally decreased with an apparent oligoclonality of the light chains (three out of six cases). One patient had a monoclonal immunoglobulin gammapathy in the ascitic fluid.

As in plasma, the retinol binding protein modification correlated perfectly with the degree of renal insufficiency. A modification in the concentration of Apo C II and III lipoproteins was seen in diabetes.

In conclusion, the protein maps of ascitic fluid were very similar to plasma maps except for two groups of proteins which were identified by microsequencing. One was seen in every ascitis, but never in plasma, and was a fragment of fibrinogen β-chain. The other was detected in high concentration only in acute pancreatitis and was found to be α_1-antitrypsin fragments.

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