Protein Complex of Fibrinogen Gamma Chain and Complement Factor H in Ovarian Cancer Patient Plasma

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Abstract. *Background: In the plasma of an advanced cancer patient, fibrinogen is sometimes increased with possible effects on red blood cells (RBCs). Materials and Methods: The plasma fraction deteriorating osmotic resistance of RBCs was separated from a patient's plasma with advanced ovarian cancer by phenyl-sepharose column chromatography and analyzed with gel filtration chromatography. Results: In the plasma fraction, we found a protein reactive against whole fibrinogen with a molecular weight higher than that of intact fibrinogen from a healthy volunteer. The-high molecular weight protein was immunoractive to an antibody against fibrinogen gamma chain but not to an antibody against alpha or beta chain. Complement factor H, identified by N-terminal sequencing of a 150-kDa protein separated from the protein, was also eluted from anti-fibrinogen gamma immunoaffinity column. Conclusion: Fibrinogen gamma chain and complement factor H were found to be bound as a protein complex in the plasma of a patient with advanced ovarian cancer.*

Fibrinogen is a large plasma protein composed of two sets of α, β and γ chains. Initially, a trimer is generated by integrating the aβ chain into the αγ complex or an α chain into the βγ complex. These trimers represent the alpha half-molecule composed of α, β and γ chains (1). Two trimers then dimerize to form a fibrinogen molecule (2). The α, β and γ chains are encoded by three independent genes

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clustered on chromosome 4. Imbalances in the intracellular levels of these chains have been observed in hepatocytes and hepatoma cells (3, 4). For example, a common feature in diseases involving hepatocytes is excess amounts of the γ chain in the plasma of patients (4).

In a previous study, a plasma fraction of a patient with advanced cancer patient plasma fraction, eluted from a phenyl-sepharose column by lowering NaCl in the buffer under 0.5 M, showed that osmotic resistance of red blood cells (RBCs), which were incubated in, had deteriorated more when compared with that from patients with benign tumors (5, 6). In the plasma fraction, we found a protein that was reactive against an anti-fibrinogen gamma antibody having a molecular weight higher than that of native fibrinogen as shown by gel filtration chromatography analysis, while sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a protein complex of fibrinogen γ chain and complement factor H.

Materials and Methods

Fractionation of plasma. Plasma samples (~4 ml) of a patient with advanced ovarian cancer and a healthy volunteer were obtained by centrifugation of their blood samples taken with heparin-coated syringes at 1,000 × *g* for 10 min. Blood sample from the patient, also used for clinical examination, was taken after informed consent according to a protocol approved by the Ethics Committee of Osaka City University Hospital. Plasma was equilibrated with 1.5 M NaCl and 20 mM phosphate buffer (pH 7.4), loaded onto a Phenyl-Sepharose Fast Flow (GE Healthcare, San Diego, CA, USA) column (16 mm diameter × 40 mm height) and fractionated by lowering the NaCl concentration from 1.5 to 0 M. Target fractions were dialyzed against phosphate-buffered saline (PBS) and 50-μl samples were incubated for 30 min at 37°C with 250 μl RBCs and 500 μl plasma from the healthy volunteer with the same blood type. Using a coil planet centrifuge (CPC) polyethylene coil, the RBCs were moved from higher osmotic pressure (150 mOsm/kg) toward lower osmotic pressure (30 mOsm/kg) by centrifugal force in a rotating apparatus

(Sanki Engineering, Ltd., Kyoto, Japan) (7) and target fractions were evaluated for their effect on the osmotic pressure at which hemolysis started to be apparent (5, 6, 8). The fraction from plasma of the patient with advanced ovarian cancer, which had an higher osmotic pressure value for initial hemolysis than the corresponding value of the control sample, was further separated by gel filtration chromatography using a Sephacryl 300 column (16 mm diameter × 600 mm height) equilibrated in 25 mM Tris-HCl (pH 8.0) and 300 mM NaCl.

Western blotting. Proteins in the collected fractions were separated by SDS-PAGE using a TGX gel (Bio-Rad, Hercules, CA, USA) and, subsequently, separated protein bands were transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare). Rabbit polyclonal antibodies against: (i) whole human fibrinogen (American Research Products, Inc., Belmont, MA, USA); (ii) alpha chain (Aviva Systems Biology, San Diego, CA, USA); (iii) beta chain (Bioss Antibody, Boston, MA, USA); or (iv) gamma chain (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were reacted in PBS with 20% FBS and 0.02% Tween 20 at appropriate dilution, washed with PBS containing 0.02% Tween 20 and, then, reacted with a rabbit antibody against peroxidase-conjugated anti-rabbit immunoglobulin goat IgG. The reactive immunocomplexes were visualized using the enhanced chemiluminescence (ECL) Prime Western blotting detection reagent (GE Healthcare) and images were recorded using a LAS 4000 Mini Image Analyzermn (GE Healthcare).

N-terminal protein sequencing. SDS-PAGE-separated proteins were blotted onto a Sequi-Blot PVDF membrane (Bio-Rad) at 1 mA/cm^2 for 2 h on two layers of blotting paper containing 300 mM Tris, 0.02% SDS and 20% (v/v) methanol and two layers of blotting paper containing 25 mM Tris, 0.02% SDS and 20% (v/v) methanol and stained with Coomassie brilliant blue R in 50% methanol and destained with 50% methanol. N-terminal amino acid sequencing was performed by Edman degradation analysis using an ABI procise 491HT instrument (Applied Biosystems, Foster City, CA, USA).

Immunoaffinity chromatography with an anti-fibrinogen γ chain antibody. Cyanogen bromide (CNBr)-activated Sepharose was coupled to an antibody against fibrinogen γ chain (Santa Cruz Biotechnology, Inc.), according to the manufacturer's protocol. Protein fractions that were eluted from the Phenyl-Sepharose column with NaCl concentration <0.5 M were separated by gel filtration chromatography. Protein samples that were identified to have a higher molecular weight than native fibrinogen by gel filtration were dialyzed against PBS, applied to an anti-fibrinogen column and eluted with 0.05% trifluoroacetic acid. Samples were pH-neutralized and concentrated by freeze-drying. The proteins in the sample were separated by SDS-PAGE using a TGX gel (Bio-Rad), transferred onto a PVDF membrane (Hybond-P, GE Healthcare), reacted with a polyclonal antibody against human complement factor H (Assaypro, St. Charles, MO, USA) and analyzed as for fibrinogen.

Results

Fractionation of plasma. Phenyl-Sepharose chromatography of the plasma sample from the patient with advanced ovarian cancer gave a fraction collected at < 0.5 M NaCl that caused RBCs' hemolysis at higher osmotic pressure (Figure 1; lane 9) when compared with the corresponding fraction derived from plasma of the healthy volunteer.

Gel filtration and western blot analysis. The plasma fraction separated by Phenyl-Sepharose chromatography and collected at <0.5 M NaCl was further separated by gel filtration chromatography. The proteins in the separated fractions were analyzed by Western blotting with an anti-whole fibrinogen antibody that detected proteins rich in the higher molecular weight range (about 400 kDa) from plasma of the patient with advanced ovarian cancer (Figure 2B, 2D; lanes 7, 8). In the protein fractions from plasma of the healthy volunteer, proteins reactive to the anti-fibrinogen antibody were located in the lower molecular weight range (about 340 kDa) (Figure 2A, 2C; lanes 3, 4), while only very weak detection of proteins reactive to this antibody was observed in the high molecular weight range fractions.

A 150-kDa protein and N-terminal sequencing. The protein fractions eluted by lowering the NaCl concentration below 0.5 M were separated by gel filtration chromatography, analyzed by SDS-PAGE and stained by silver staining (Figure 3A). A 15 kDa protein in the plasma fractions from the patient with advanced ovarian cancer was identified (Figure 3A; lane 7, indicated with an arrowhead).

We also analyzed the molecular composition of fibrinogen chains by Western blotting using antibodies against fibrinogen α, β and γ chains. The analysis revealed that fibrinogen γ chain was rich (Figure 3D; lanes 6 and 7) but the α and β chains of fibrinogen were not found in the same lane of the protein with a molecular weight of 150 kDa (Figure 3B and C; lanes 6 and 7). N-terminal sequencing analysis of the 150 kDa protein identified to contain the amino acid sequence of ED_NELP (corresponding to amino acid 19 to 25 of human complement factor H in which amino acid 21 cysteine was not detected because of Edman degradation analysis); the 150-kDa protein was identified as human complement factor H.

Immunonaffinity chromatography. To confirm whether fibrinogen γ chain and complement factor H are forming a complex or eluted simultaneously by phenyl-sepharose column chromatography and by gel filtration chromatography, we carried out anti-fibrinogen gamma immunoaffinity chromatography. A 150-kDa protein, reactive to an anti-human complement factor H antibody, was eluted from the anti-fibrinogen γ column (Figure 4C; indicated with an arrowhead) as observed by western blot analysis. From these results, we conclude that complement factor H and the fibrinogen γ chain form a stable complex under the conditions examined.

Discussion

Fibrinogen is not only a coagulation factor but also an acute-phase protein (9). Fibrinogen expression is regulated by inflammatory mediators like interleukin-6 (10, 11), with the intact molecule existsing as a 340-kDa plasma protein;
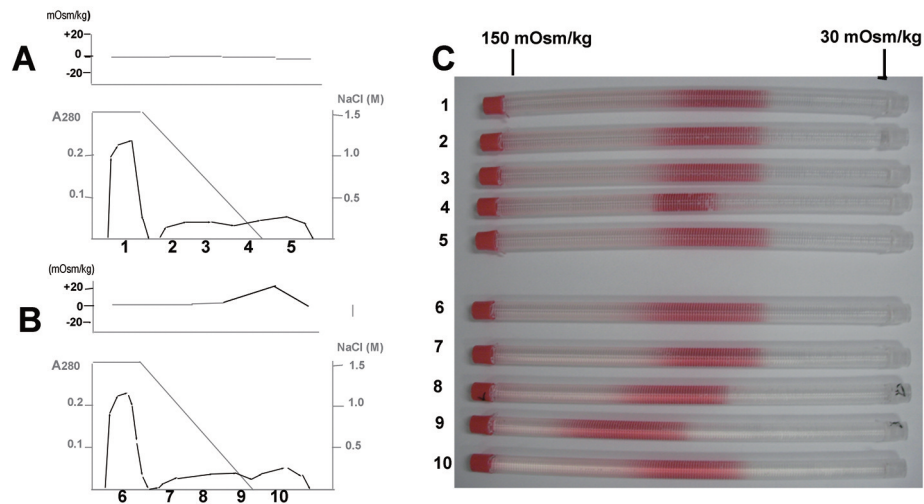
Figure 1. *Column chromatography of plasma and the effect of osmotic pressure on RBC lysis. Plasma from the healthy volunteer (A) and the patient with advanced ovarian cancer (B) were separated by phenyl-sepharose column chromatography. The isolated fractions were incubated with red blood cells (RBCs) and the effects of osmotic pressure on RBC lysis were examined in the coil planet centrifuge (CPC) (C). The plasma fraction from the patient with advanced ovarian cancer, eluted at NaCl concentration below 0.5 M (lane 9), was found to start RBCs' lysis at higher osmotic pressure when compared with that from the healthy volunteer. The differences of osmotic pressure at which RBCs start to be lysed after incubation with the plasma fraction and PBS are indicated in each upper graph.*
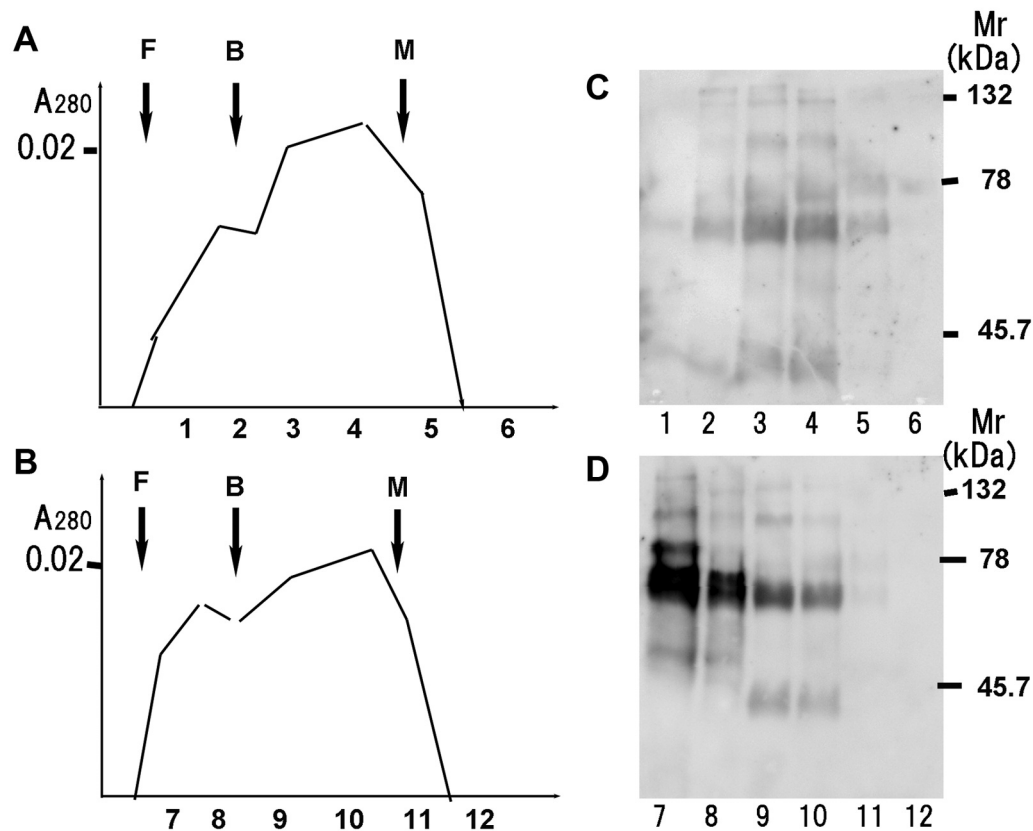

Figure 2. *Gel filtration chromatography of phenyl-sepharose fraction collected at <0.5 M NaCl. The Phenyl-Sepharose fraction collected at <0.5 M NaCl from the healthy volunteer (A) or the patient with advanced ovarian cancer (B) was separated by Sephacryl S300 gel filtration and the proteins in fractions 1-12 were analyzed by western blotting with an anti-whole fibrinogen polyclonal antibody. Standard molecular weight markers were ferritin (Mr 440,000) (F), bovine serum albumin (Mr 76,000) (B) and myoglobin (Mr 17,800) (M); these proteins eluted at positions marked F, B and M on the chromatogram.*
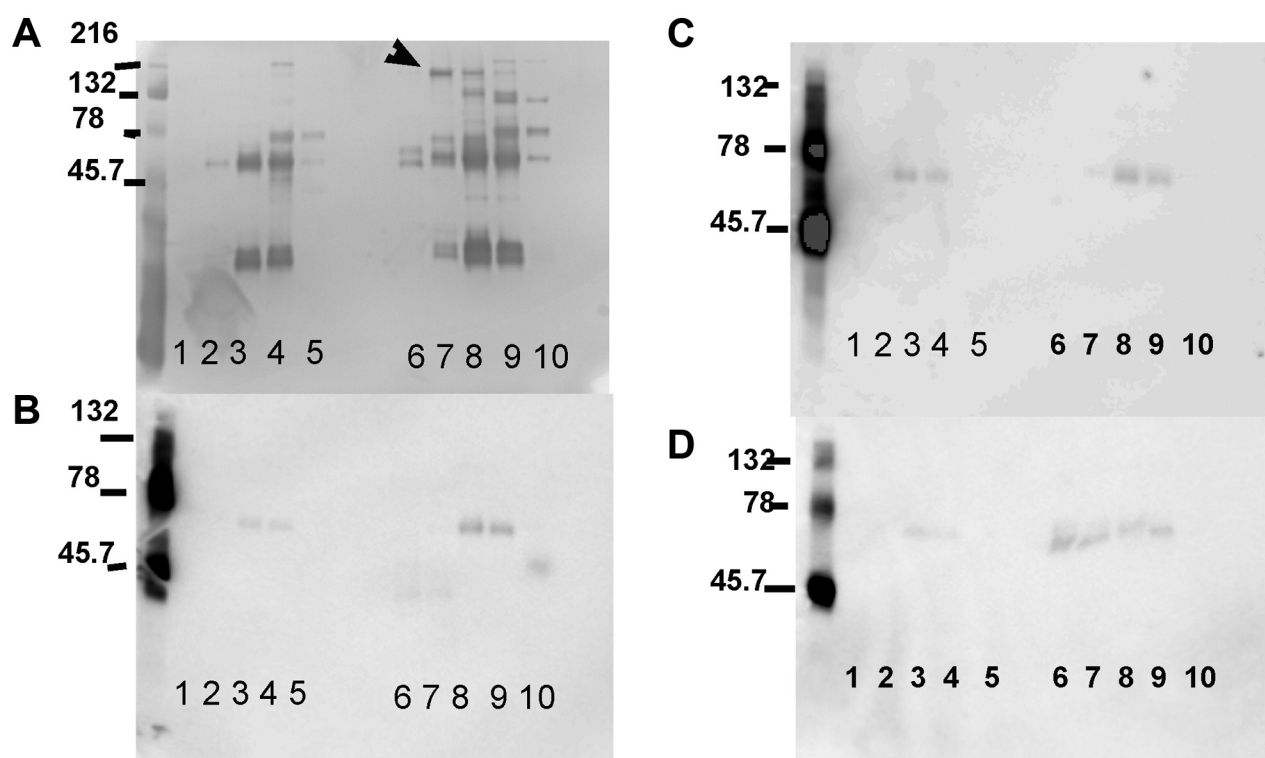
Figure 3. *Western blot analysis of proteins separated by gel filtration chromatography of the phenyl-sepharose fraction collected at <0.5 M NaCl. The phenyl-sepharose fractions collected at <0.5 M NaCl from plasma of the healthy volunteer (fractions 1-5) and the patient with advanced ovarian cancer (fractions 6-10) were separated by Sephacryl S300 gel filtration and the proteins isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were stained with silver stain (A). For western blotting an anti-fibrinogen α polyclonal antibody (B), anti-fibrinogen β polyclonal antibody (C) and anti-fibrinogen γ polyclonal antibody (D) were used. A 150-kDa protein (indicated with an arrowhead) and the fibrinogen γ chain were identified in the higher molecular weight range of the phenyl-sepharose <0.5 M NaCl fraction derived from the plasma of the patient with advanced ovarian cancer.*

however, a C-terminal α chain proteolysis product with 305 kDa molecular weight is also found in plasma (11, 12). Although fibrinogen inhibits acute-phase damage like thermal induced protein aggregation (13), homodimers of fibrinogen γ chain have rouleaux formation-inducing effects on RBCs (14).

As each fibrinogen chain is synthesized from an individual gene, excess production of a particular chain due to dysfunctional gene regulation may cause self-association and the generation of homo-polymers (15). An excess of γ chains has been reported in cultured cells (4).

Phenyl-sepharose chromatography was used to identify a fraction rich in fibrinogen γ chain eluted at a NaCl <0.5 M, indicating that this chain is hydrophobic in nature. RBCs' membrane may be sensitive to hydrophobic proteins and the CPC coil represents a potential tool to test the effects of proteins on RBCs (8). Regrettably, the manufacturer stopped production of the CPC coil and we could no longer examine at which point of osmotic pressure hemolysis begins to occur under various conditions in different samples. We are,

currently, searching for alternative methods to examine the osmotic resistance of RBCs.

Complement factor H is a soluble protein that is a member of the regulators of the complement system. In particular, complement factor H is involved in normal recognition of self-cell markers and the regulation of C3 activation (17). RBCs are targets of complement activation unless they are protected by three major regulatory proteins: CD35, CD55 and CD59. CD55 and CD59 are present on the surface of RBCs and prevent membrane attack by the complement system (18, 19). Complement factor H is also a protector of RBCs against attack from C3b or other complement-related factors (20). We considered that excessive fibrinogen γ chain inhibits the function of complement factor H by hetero-polymer formation and RBCs may be easily exposed to complement attack.

Conflicts of Interest

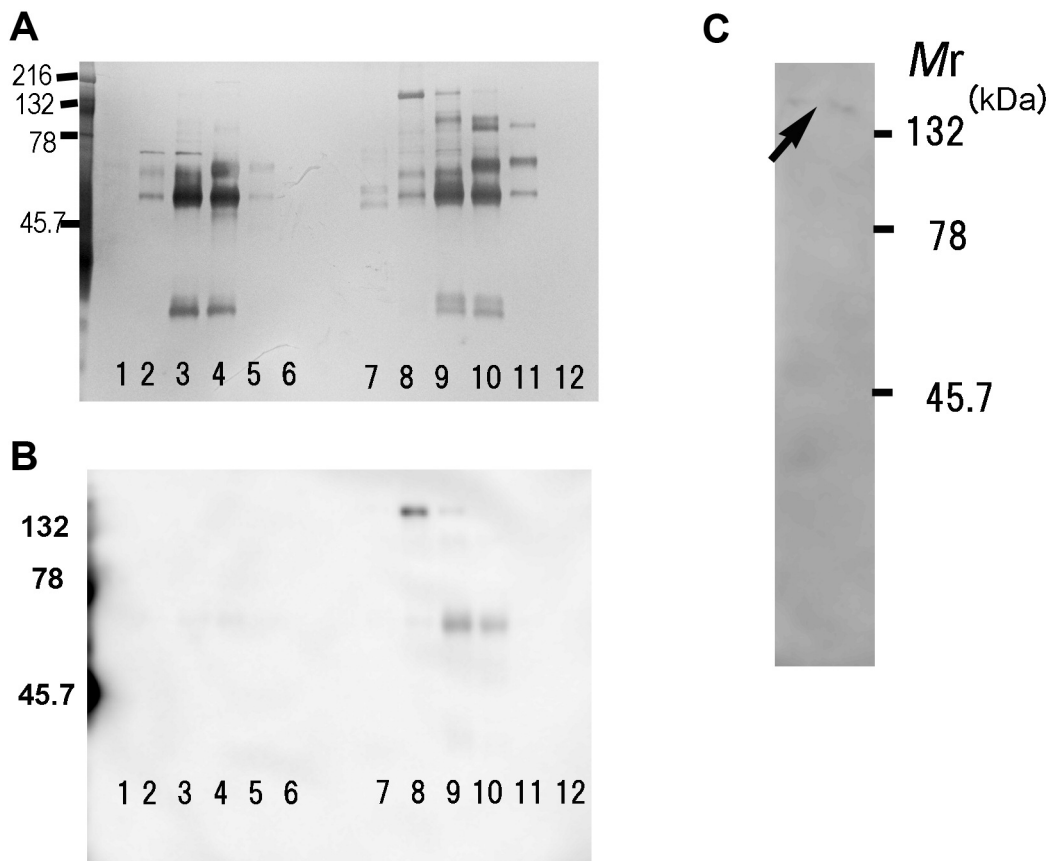The Authors declare no conflict of interest.

Figure 4. *SDS-PAGE and western blot analysis of proteins separated by gel filtration chromatography of the phenyl-sepharose <0.5 M NaCl fractionation and eluted from anti-fibrinogen γ immunoaffinity column. A protein with molecular weight of 150 kDa was identified by silver staining (A) and enhanced chemiluminescence (ECL) western blotting detection using an anti-complement factor H antibody (B). The 150-kDa protein (indicated with an arrowhead) eluted from the immunoaffinity column against the fibrinogen γ chain was also detected by ECL western blotting using an anti-complement factor H antibody (C).*

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