High-level expression and preparation of recombinant human fibrinogen as biopharmaceuticals

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Fibrinogen is a large and complex glycoprotein containing two sets of each of three different chains (α , β and γ). There have been no reports of high-level expression of fibrinogen at commercial levels using mammalian cultured cells such as CHO cells because of the difficulty in highly expressing a protein with such a complex structure. We achieved high-level (1.3 g/l or higher) expression of recombinant human fibrinogen using CHO DG44 cells by optimizing the expression system and culture conditions. We also succeeded in establishing a high-recovery preparation method for recombinant fibrinogen that rarely yields degraded products. To characterize the properties of the recombinant human fibrinogen, we performed SDS-PAGE; western blotting of the α , β and γ chains using specific antibodies and scanning electron microscopy observations of fibrin fibres. We also evaluated the functional equivalence between recombinant fibrinogen and plasma fibrinogen with respect to the release of fibrinopeptides initiated by thrombin and its cross-linking properties. The basic properties of recombinant fibrinogen showed no apparent differences from those of plasma fibrinogen. Here, we report the development of methods for the culture and preparation of recombinant human fibrinogen of satisfactory quality that can be scaled up to the commercial level.

Keywords: biopharmaceuticals/CHO DG44/high-level expression/high-recovery preparation/recombinant human fibrinogen.

Abbreviations: dFBS, dialyzed fetal bovine serum; DTT, dithiothreitol; PDL, population doubling level; PNGase F, Peptide-N-glycosidase F; SD, solvent/detergent; SEC, size exclusion chromatography; SEM, scanning electron microscopy; TFA, trifluoroacetic acid; TNBP, Tri-N-butyl phosphate; UV, ultraviolet; WB, western blotting.

Many blood plasma products such as albumin, globulin and coagulation factors, including fibrinogen and thrombin, are used therapeutically. Although recombinant albumin, several monoclonal antibodies and some coagulation factors are available as biopharmaceuticals (1–3), production of fibrinogen for this purpose has not been successful.

Fibrinogen is a clotting factor that acts in the final stage of the blood coagulation cascade and is converted into a fibrin monomer by activated thrombin. The fibrin monomers then polymerize in the presence of coagulation factor XIII (FXIII) and calcium to form fibrin polymers, which are precipitated as fibrin fibrils in tissue (4). These insoluble fibrin clots and aggregated platelets constitute a thrombus, which leads to haemostasis. Fibrin sealants based on this clotting action have been used for more than 20 years as surgical haemostatic and sealing agents (5, 6).

Fibrinogen, a non-spherical protein with a large molecular size of 340 kDa, is susceptible to physical shearing and degradation by proteases in the manufacturing process because of its structural characteristics. Degraded fibrinogen cannot form a strong polymer structure even when transformed to fibrin, resulting in the failure of haemostasis (7–9). Although fibrinogen is not degraded, if it is not controlled with appropriate pH or salt concentrations, its function may not be sufficiently expressed (10, 11).

Similar to other plasma derivatives, human plasma-derived fibrinogen as a pharmaceutical product is constantly exposed to the risk of contamination with materials such as unknown viruses or prions (12). Thus, development of a recombinant human fibrinogen as a pharmaceutical product is desirable for improvement of safety.

Recombinant fibrinogen has been expressed in systems such as in the milk of transgenic animals (13–17) and in mammalian cells (18–20). Each expression system has both merits and demerits. Transgenic animal milk expression systems are attractive systems that are particularly useful from the perspective of the quantity expressed. However, they are incompatible with the recent trend of avoiding the use of animal-derived ingredients, and it is difficult to commercialize them because they require special animal-raising facilities. Cultured cell-based expression systems afford important benefits such as achievement of safety and reproducibility by quality control of the master cell bank and availability of reliable experience with using commercial antibody drugs. Despite these benefits, there are no reports of applications of this method that express recombinant fibrinogen on a commercial scale because achieving large-scale expression would be difficult.

Furthermore, there are problems in the development of efficient manufacturing processes for recombinant fibrinogen. In general, recombinant proteins of sufficient purity for safe administration to patients are manufactured by complex purification processes (21). For biopharmaceuticals, achieving high purity with the fewest possible processes, along with limiting degradation and yield decline, is a challenge in the development of recombinant fibrinogen.

Here, we describe the high-level expression of recombinant human fibrinogen, which is achieved by adopting an expression system previously reported by us (22), and the establishment of a high-recovery manufacturing process that can be easily scaled up to commercial level while ensuring the safety and quality of the expressed product.

Materials and Methods

Materials

CHO DG44 cells, CD DG44 medium, CD OptiCHO medium, 200 mM L-glutamine, 100× antibiotic–antimycotic (10,000 U/ml penicillin, 10,000 µg/ml streptomycin and 25 µg/ml amphotericin B), Opti-MEM, Lipofectamine 2,000 CD reagent, dialyzed fetal bovine serum (dFBS) and POROS HQ 50 µm Bulk Media were purchased from Life Technologies (Carlsbad, CA). G418 disulfate salt solution, MTX and general reagents were purchased from Sigma–Aldrich (St. Louis, MO). YMM medium was originally prepared on the basis of MEM alpha medium, and 302/T50 media were originally prepared on the basis of EX-CELL302 medium (SAFC Biosciences, Lenexa, KS). Polysorbate 80 was obtained from NOF Corporation (Tokyo, Japan). Tri-N-butyl phosphate (TNBP) was obtained from Merck Millipore (Darmstadt, Germany). Capto MMC resin was purchased from GE Healthcare (Uppsala, Sweden). Other general reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan). Human plasma-derived fibrinogen and human plasma-derived FXIII were purchased from Enzyme Research Laboratories (South Bend, IN). Human plasma-derived α -thrombin was from Haematologic Technologies (Essex Junction, VT). Anti-fibrinogen monoclonal antibodies were derived from hybridomas produced by the fusion of mouse myeloma cells and lymph node

lymphocytes from a plasma-derived fibrinogen immunized mouse. TMB substrate and BCIP/NBT phosphatase substrate were obtained from Kirkegaard and Perry Laboratories (KPL, Gaithersburg, MD). Peptide-N-glycosidase F (PNGase F) was purchased from Roche (Mannheim, Germany).

Construction of the expression plasmid

For a human fibrinogen gene, pairs of primers consisting of a Kozak sequence and an added *SalI* restriction site for an α chain, β chain and γ chain were prepared (FbgA-1: 5'-ccccaagctt gtcgacgcca ccatgttttc catgaggatc gtctg-3', FbgA-2: 5'-ccatcgatgg atccgctgac ttactagggg gacaggggaag gcttcccaa aggagaagtg tggatac-3'; FbgB-1: 5'-ccccaagctt gtcgacgcca ccatgaaaca ttattattg ctactattgt gtgtttttct agttaagtcc caa-3', FbgB-2: 5'-cggaattctg atcagtcgac ttactattgc tgtgggaaga agggcctgat ctctactc atctctc-3'; FbgG-1: 5'-ccccaagctt gtcgacgcca ccatgagttg gtccttcac ccccggaatt taattc-3', FbgG-2: 5'-cggaattcgg atccgctgac ttattaaacg tctccagct gtttgctccc-3'), and PCR was performed using the Advantage HF-2 PCR kit (BD Biosciences, San Jose, CA) and human liver Marathon-Ready cDNA (BD Biosciences) as a template according to the manufacturer's protocol. The *SalI* fragment of each of the three genes (α , β and γ chains) was inserted into a CAGG expression cassette, which was enclosed in a 582-bp fragment (Ars insulator) (23, 24). The CAGG expression cassette comprises the CAG promoter and the rabbit β -globin poly(A) signal (25). The resulting plasmid, SPNFH-ArsFF-Fbg $\alpha\gamma\beta\gamma$ (Fig. 1), comprised four CAGG expression cassettes, resulting in a constituent ratio of 1:1:2 for the respective genes of the α , β and γ chains. It also contained the expression cassette for the dhfr gene and the neomycin resistance gene (neo) in mammalian cells.

Generation and screening of recombinant human fibrinogen high-producing cell lines

The CHO DG44 cells were seeded in a 125-ml Erlenmeyer shaker flask in CD DG44 medium supplemented with 8 mM L-glutamine and 1× antibiotic–antimycotic and incubated on an orbital shaker platform rotating at 80 rpm. On the day of transfection, the cells were resuspended at 2.0×10^6 cells/ml in 2 ml of YMM medium supplemented with nucleic acid and incubated for 1 h in a 6-well plate without shaking. Following this step, 510 µl of the complex mixed with 4.0 µg of linearized SPNFH-ArsFF-Fbg $\alpha\gamma\beta\gamma$ in 250 µl of Opti-MEM and 10 µl of Lipofectamine 2,000 CD reagent in 250 µl of Opti-MEM was added dropwise into the 6-well plate and incubated for 4 h without shaking. Next, 1 ml of YMM medium supplemented with 10% dFBS was added and further incubated overnight. On the next day, the cells were resuspended in YMM medium supplemented

Fig. 1 Map of the expression plasmid SPNFH-ArsFF-Fbg $\alpha\gamma\beta\gamma$ for human fibrinogen. The CAGG expression cassettes containing human fibrinogen α , β and γ genes enclosed by Ars insulators were arranged in the order of α , γ , β and γ cassettes.

with 500 µg/ml G418, 100 nM MTX, 2 mM L-glutamine and 1× antibiotic–antimycotic (selective medium) and divided equally into five 96-well plates. After culture for 2–3 weeks, the transfectants that emerged in each well were multiplied and maintained in the selective medium. For selection of recombinant human fibrinogen high-producing cell lines from many transfectants, batch culture was performed in 6-well plates. In brief, candidate cells (6×10^5 cells) resuspended in 3 ml of 302/T50 medium supplemented with 2 mM L-glutamine and 1× antibiotic–antimycotic were cultured for 7–10 days and their expression level was measured by ELISA using rabbit anti-human fibrinogen polyclonal antibody (DAKO, Carpinteria, CA). ELISA was performed using a method similar to that described previously (22). All cell culture experiments were performed in a 37°C incubator with a humidified atmosphere of 5% CO₂ in air.

Gene amplification by MTX and cloning of recombinant human fibrinogen high-producing cells

Target gene amplification was performed by culture of the selected cells in 6-well plates in 2 ml of CD OptiCHO medium lacking hypoxanthine and thymidine and supplemented with 200, 500, or 1,000 nM MTX, 2 mM L-glutamine and 1× antibiotic–antimycotic. In brief, the cells were seeded at a density of 2×10^5 cells/ml in 2 ml of the medium containing 200 nM MTX in 6-well plates and cultured without shaking for 3–4 days. If cell viability remained > 80% without a clear declining trend, a passage was performed with the next higher concentration of MTX. In contrast, if it showed a declining trend or did not recover to at least 80%, MTX was maintained at the same concentration. Clonal selection by limiting dilution was performed in YMM medium supplemented with 10% dFBS, 2 mM L-glutamine and 1× antibiotic–antimycotic on 96-well plates with a density of 0.5 cells per well.

Culture for assessment

To select human fibrinogen high-producing clones, we performed batch culture and/or fed-batch culture in 6-well plates. In brief, 8×10^5 cells resuspended in 4.0 ml of 302/T50 medium supplemented with 2 mM L-glutamine and 1× antibiotic–antimycotic were cultured in 6-well plates on an orbital shaker platform rotating at 80 rpm, and the number and proportion of viable cells and the expression level of recombinant human fibrinogen were monitored over time. To assess the stability of the cells, 2.5×10^5 cells

resuspended in 1.5 ml of 302/T50 medium supplemented with 2 mM L-glutamine and 1× antibiotic–antimycotic were cultured with passage every 3–4 days into fresh medium in 6-well plates as a simple and static batch culture, and SPR was monitored to a population doubling level (PDL) of at least 40.

Purification of fibrinogen

Recombinant human fibrinogen was purified from the culture supernatant of human fibrinogen-expressing CHO DG44 cells. For harvesting the cell culture fluid and removing the cell debris, microfiltration membranes were used. The filtered culture supernatant was applied to a Capto MMC column equilibrated with 20 mM phosphate buffer (pH 7.0) containing 120 mM NaCl. The column was washed with equilibration buffer, and bound fibrinogen was eluted from the column with 200 mM arginine buffer (pH 8.5) containing 500 mM NaCl. The Capto MMC-eluted fibrinogen solution was irradiated with 150 J/m² of 254 nm ultraviolet C (UV-C) with UViviatec lab (Sartorius, Göttingen, Germany) for viral inactivation. Buffer exchange in 12.5 mM arginine buffer (pH 8.5) was performed by ultrafiltration. For solvent/detergent (SD) viral inactivation, buffer-exchanged recombinant human fibrinogen solution was adjusted to concentrations of 0.3% TNBP and 1% polysorbate 80 and incubated for 2 h at 20°C. SD-treated fibrinogen solution was applied to a POROS 50HQ column equilibrated with 12.5 mM arginine buffer (pH 8.5) and washed with 12.5 mM arginine buffer (pH 8.5) containing 100 mM NaCl. Recombinant human fibrinogen was eluted from a POROS 50HQ column with 12.5 mM arginine buffer (pH 8.5) containing 270 mM NaCl. Finally, the fibrinogen eluate was replaced with 10 mM arginine buffer (pH 8.5) containing 130 mM NaCl. The concentrations of the fibrinogen solutions at each step were quantified by size exclusion chromatography (SEC).

SDS-PAGE and western blotting analysis

Samples were prepared for electrophoresis in Laemmli (26) sample buffer in the absence or presence of 10 mM dithiothreitol (DTT) and separated on SDS-PAGE using AE-6500 Dual Mini Slab (ATTO, Tokyo, Japan). Proteins were stained with Quick CBB (WAKO, Tokyo, Japan). Electrophoretic transfer onto PVDF membranes was performed using a Rapid Semi-Dry blotter (ATTO). Membranes were incubated with anti-fibrinogen mouse monoclonal antibodies, followed by alkaline phosphatase-labelled goat anti-mouse

Fig. 2 Cell proliferation and fibrinogen expression. (A) Cell density (triangles) and viability (circles) were monitored for 16 days. (B) Expression levels of recombinant fibrinogen were quantified by ELISA (diamond symbols). (C) Recombinant fibrinogens in culture supernatants of Days 7–15 were estimated using 10% SDS-PAGE under reducing conditions and CBB staining. Fibrinogen α chain (α), fibrinogen β chain (β) and fibrinogen γ chain (γ) are shown by arrow heads.

IgG (Merck Millipore). To visualize enzyme activity, signals were developed with BCIP/NBT phosphatase substrate.

Glycosidase digestion of fibrinogen

Fibrinogen (1.6 mg/ml) in TBS [50 mM Tris-HCl (pH 7.5), 150 mM NaCl] was digested with 100 U/ml PNGase F at 37°C overnight. Glycosidase-digested fibrinogens were analysed by SDS-PAGE and western blotting using fibrinogen chain-specific antibodies, and mass spectra of fibrinogens were generated by LC/MS.

LC/MS analysis of reduced fibrinogen

Fibrinogen was reduced with 6 M guanidine and 10 mM DTT. Reduced fibrinogen was separated using a linear gradient of 10–45% acetonitrile containing 0.1% v/v trifluoroacetic acid (TFA; Thermo Fisher Scientific, Waltham, MA) on a BEH300 C4 reversed-phase column using a Waters Acquity I-Class UPLC instrument connected to a Waters Synapt G2-Si HDMS mass spectrometer (Waters Corporation, Milford, MA). The mass spectrometer was operated in the positive-ion mode with a mass range of 500–4,000 Da.

Fibrin polymerization turbidity curves

Polymerization of fibrinogen (at 2 mg/ml) was initiated by addition of thrombin (0.05 U/ml) in TBS containing 10 mM CaCl₂ and 0.5 U/ml FXIII. Polymerization of fibrin was evaluated by measurement of the change in turbidity over time at 450 nm using a U-3900 spectrophotometer (Hitachi, Tokyo, Japan). Measurements were performed at 25°C in a 1.5-ml semimicro UV cuvette, and data were collected at a sampling interval of 0.2 s.

Scanning electron microscopy

Fibrin clots were formed as described in the previous section and were allowed to mature for 2 h after thrombin addition. The samples were fixed in 1% OsO₄, dehydrated in ethanol, frozen at –80°C and broken apart. These samples were placed in *t*-butyl alcohol, lyophilized and coated with a layer of sublimated OsO₄ using an osmium plasma coater, and fibrin fibre structures were examined under a field-emission scanning electron microscope (JSM-6320F; JEOL, Tokyo, Japan).

Release of fibrinopeptide A and B

Release of fibrinopeptide A and B from fibrinogen was measured using reverse-phase HPLC. Digestion of fibrinogen with thrombin was performed in TBS containing 10 mM CaCl₂ at 25°C under cross-linking conditions with FXIII. The reactions were initiated by addition of thrombin (0.5 U/ml) to a mixture containing fibrinogen (1 mg/ml) and FXIII (1 U/ml). The reactions were stopped at specified times by addition of equal volumes of 0.1 M HCl containing 8 M guanidine HCl. Fibrin clots were dissolved using an UltraS homogenizer (VP-5S; TAITEC, Saitama, Japan) and filtered through 0.45-μm spin filters. Fibrinopeptides were separated using a linear gradient of 10–45% acetonitrile containing 0.1% v/v TFA on a ZORBAX 300SB C18 reversed-phase column using an Agilent 1,260 infinity HPLC instrument (Agilent Technologies, Santa Clara, CA).

Cross-link formation analysis

Cross-linking reactions were performed in TBS containing 10 mM CaCl₂ at 25°C. The reactions were initiated by addition of thrombin (1 U/ml) to a mixture containing fibrinogen (2 mg/ml) and FXIII (1 U/ml). The reactions were stopped at specified times by addition of Laemmli sample buffer containing 4 M urea, followed by boiling. The samples were then subjected to 10% SDS-PAGE, followed by western blotting.

Viscoelasticity measurement

Fibrin clots were prepared in 2-ml round-bottom plastic tubes under the conditions described in the previous section. Viscoelasticity of the fibrin clots was measured using an EZ-Test EZ-S table-top universal tester (SIMADHU, Tokyo, Japan) with a 5-N load cell and 4-mm compression plunger.

Results

Establishment of a stable cell line

A stable cell line was generated from CHO DG44 cells by the protocols described in the Materials and Methods section. After transfection of the human fibrinogen gene, the culture supernatants of G418-resistant cells in 96-well plates were assayed for the expression level of recombinant human fibrinogen. Cells from the well (HM08-4_84) that

Fig. 3 SDS-PAGE and western blotting (WB) of recombinant fibrinogen. (A) Under reducing and non-reducing conditions, recombinant fibrinogen and plasma fibrinogen were estimated by 5–20% gradient SDS-PAGE stained with CBB. (B) Under reducing conditions, α, β and γ chains are shown by WB using fibrinogen chain-specific antibodies. pFbg, plasma fibrinogen; rFbg, recombinant fibrinogen.

showed high fibrinogen concentrations by ELISA were subjected to gene amplification by MTX and to clonal selection by limiting dilution (twice). In addition, SPR (20 pg/cell/day) of the clone remained virtually constant during 40 PDLs, and the nucleic acid sequence of fibrinogen genes extracted from the cells following 40 PDLs was identical to the designed sequence (data not shown). This clone accordingly became a candidate for a master cell bank in future manufacturing.

Preparation of recombinant human fibrinogen

Stable cell lines expressing ~1.1 mg/ml of recombinant human fibrinogen were established in shaker flasks. For large-scale preparation, scale-up cultivation was performed in a 5-l fermenter. Cell density and viability are shown in Fig. 2A. The culture was terminated at 16 days when the cell viability was <~70%. The

expression level of recombinant human fibrinogen reached 1,319 µg/ml at the end of the culture (Fig. 2B). As shown on SDS-PAGE (Fig. 2C), the intensity of bands representing the three fibrinogen chains (α , β and γ) almost evenly increased with culture time and degraded fibrinogens were not detected. The band shown in the gel front was not derived from a fibrinogen but was derived from host cell proteins, which were also detected in the culture supernatant of host cell (data not shown). The same results were confirmed in a 400-l fermentation culture (data not shown). The recombinant product from the 400-l cultivation was purified as described in the Materials and Methods section. The final product exhibited a high yield ($\geq 50\%$, quantified by SEC) and high purity [$\geq 97\%$, quantified by ELISA for host cell proteins, as described previously (22)].

Fig. 4 Carbohydrate analysis of recombinant and plasma fibrinogens. (A) Molecular weight changes of fibrinogens treated with peptide-N-glycosidase F (PNGase F) were evaluated by 5–20% gradient SDS-PAGE stained with CBB and WB using fibrinogen chain-specific antibodies. Samples 1 and 2 are plasma fibrinogen treated without and with PNGase F, respectively. Samples 3 and 4 are recombinant fibrinogen treated without and with PNGase F, respectively. (B) Chains of reduced fibrinogen were separated and analysed by LC/MS. Data of deconvoluted mass spectra for each of the fibrinogen chains are shown, and differences in the molecular weight following PNGase F treatment are indicated in parentheses. pFbg, plasma fibrinogen; rFbg, recombinant fibrinogen.

Structural analysis of purified recombinant human fibrinogen

Purified recombinant human fibrinogen was analysed by SDS-PAGE and western blotting. Purified recombinant fibrinogen and plasma-derived fibrinogen showed bands at ~68, 55 and 48 kDa on SDS-PAGE under reducing conditions and bands of similar sizes under non-reducing conditions (Fig. 3A).

These bands were identified by western blotting under reducing conditions using chain-specific monoclonal antibodies, as shown in Fig. 3B. Slight differences, not visible by CBB staining, were observed in the blots using anti- α chain, anti- β chain and anti- γ chain antibodies. The band size of the α chain of recombinant fibrinogen was slightly greater than that of the α chain of plasma-derived fibrinogen, whereas the size of the β and γ chains were slightly smaller.

To characterize the carbohydrate chain present on recombinant fibrinogen, purified recombinant fibrinogen and plasma fibrinogen were treated with PNGase F. Fibrinogens treated with and without PNGase F were analysed by SDS-PAGE under reducing conditions. As shown in Fig. 4A, changes in band mobility showed that both β and γ chains had N-linked carbohydrate chains in recombinant fibrinogen, similar to plasma fibrinogen. The motilities of the α chains in the recombinant and plasma fibrinogens were not altered after PNGase F treatment.

To evaluate changes in the molecular weight after PNGase F treatment, fibrinogens treated with and without PNGase F were reduced using DTT, separated by HPLC and analysed by mass spectrometry. Figure 4B shows deconvoluted mass data of reduced fibrinogen chains separated and analysed by LC/MS. Recombinant fibrinogen and plasma fibrinogen showed similar results. The molecular weights of the α chains obtained from the deconvoluted mass spectral

data were unchanged; however, differences were observed in the β and γ chains.

The α chain of recombinant fibrinogen showed a larger size than that of plasma fibrinogen, and both fibrinogens showed a lower value than the theoretical molecular weight (α : 67,660 Da). The β chains of glycosidase-treated recombinant and plasma fibrinogens were of similar size, and the γ chains were of exactly the same size. The molecular weights of β and γ carbohydrate-cleaved chains were close to the theoretical values (β : 52,314 Da, γ : 46,467 Da). Although the N-terminal degradation of α chains was not observed in recombinant and plasma fibrinogens, the C-terminal amino acids of both fibrinogens were slightly deleted (data not shown). The α chain of plasma fibrinogen mainly showed a molecular weight of 66,132 Da as reported previously (27), and the C-terminal deletion of recombinant fibrinogen was slight degree in comparison with that of plasma fibrinogen (data not shown).

In view of the difference in the molecular weight between glycosidase-treated recombinant and plasma fibrinogens, it was confirmed that different carbohydrate chains had been added. Carbohydrate chains cleaved from the β and γ chains of plasma fibrinogen by glycosidase treatment were of the biantennary digalactosylated disialylated type (A2G2S2), biantennary digalactosylated monosialylated type (A2G2S1) and biantennary digalactosylated non-sialylated type (A2G2), as reported previously (28), and the molecular weight changes were similar to the theoretical molecular weights of the three carbohydrate chains (2,222, 1,931 and 1,640, respectively).

The four carbohydrate chains cleaved from recombinant fibrinogen by glycosidase treatment were of the core fucosylated biantennary digalactosylated type (FA2G2), core fucosylated biantennary monogalactosylated type (FA2G1), core fucosylated biantennary

Fig. 5 Polymerization evaluation of recombinant fibrinogen by monitoring changes in turbidity. Polymerization was initiated by addition of thrombin (0.05 U/ml) at time 0 under FXIII-containing conditions (0.5 U/ml) to 2 mg/ml of recombinant fibrinogen (broken line) or plasma-derived FXIII-depleted fibrinogen (solid line), and polymerization was evaluated by monitoring changes in turbidity at 450 nm for 30 min ($N = 2$).

non-galactosylated type (FA2G0) and high mannose type, as reported for proteins expressed in CHO cells (29). The molecular weight changes were consistent with the theoretical molecular weights of the four carbohydrate chains (1,786, 1,624, 1,462 and 1,234, respectively).

Functional analysis of purified recombinant human fibrinogen

To evaluate the differences in the polymer structure of recombinant fibrinogen and plasma fibrinogen during fibrin clotting initiated by thrombin, polymerization of

Fig. 6 SEM images of fibrin fibres. Fibrin clots were formed under FXIII-containing conditions (0.5 U/ml) by addition of thrombin (0.05 U/ml) to recombinant or plasma-derived FXIII-depleted fibrinogen (2 mg/ml). Clots were fixed, dehydrated, lyophilized, coated with osmium and imaged by SEM. The magnification bar represents 1 µm. pFbg, plasma fibrinogen; rFbg, recombinant fibrinogen.

Fig. 7 Release of fibrinopeptides A and B from recombinant fibrinogen and plasma fibrinogen. The release of fibrinopeptide A is shown in panel A and that of fibrinopeptide B is shown in panel B. Each of the fibrinopeptides was quantitated by HPLC using absorbance at 210 nm ($N = 2$). Circles represent plasma fibrinogen, and diamonds represent recombinant fibrinogen.

Fig. 8 Changes in the form of fibrinogen chains in cross-linking reactions and viscoelastic evaluation of fibrin clots. At time 0, thrombin (1 U/ml) was added to a mixture of FXIII (1 U/ml) and plasma or recombinant fibrinogen (2 mg/ml). The reactions were stopped at set times (0, 5, 10 and 30 min) by addition of SDS-PAGE buffer containing 4 M urea and boiling. Fibrinogen Samples 1–8 were electrophoresed on 10% SDS-PAGE under reducing conditions and stained with CBB (A), followed by WB using anti- α chain (B), anti- β chain (C; anti-fibrinopeptide B) and anti- γ chain (D) antibodies. Lanes 1–4 are plasma fibrinogen and 5–6 are recombinant fibrinogen. Lanes 1 and 5 are 0 time samples. Lanes 2 and 6 are samples after 5 min. Lanes 3 and 7 are samples after 10 min. Lanes 4 and 8 are samples after 30 min. (E) Fibrin clots were reacted for 30 min without stopping the reaction, and the elastic modulus was measured using an EZ-Test EZ-S table-top universal tester ($N = 5$). pFbg, plasma fibrinogen; rFbg, recombinant fibrinogen.

fibrin was monitored by evaluating turbidity changes and fibre structures were observed by scanning electron microscopy (SEM). No great difference in turbidity changes in the polymerization of recombinant fibrinogen and plasma fibrinogen was observed (Fig. 5). In SEM observation, although a slight difference in fibre thickness was observed, the fibre structures were essentially identical (Fig. 6).

Under clotting conditions with FXIII, differences in fibrinopeptide release and the cross-linking behaviour of recombinant fibrinogen and plasma fibrinogen were evaluated. As shown in Fig. 7, the release rate of fibrinopeptide A in recombinant fibrinogen was slightly higher than that in plasma fibrinogen, and the release rate of fibrinopeptide B in recombinant fibrinogen was slightly lower than that in plasma fibrinogen for up to 3 min immediately after the reaction.

No differences were observed in the remaining α chain between recombinant and plasma fibrinogen (Fig. 8A and B), and the remaining content of the β chain, which was detected with anti-fibrinopeptide B, also showed a similar pattern in recombinant and plasma fibrinogen (Fig. 8C).

As shown in Fig. 8A, B and D, similar changes in α chain polymerization and γ chain dimerization were detected in both fibrinogens. Thus, no marked difference between the cross-linking behaviours of recombinant and plasma fibrinogen was observed.

The elasticity of recombinant fibrinogen was lower than that of plasma fibrinogen, although the difference was not significant and the behaviours of the fibrinogens were similar (Fig. 8E).

Discussion

The goal of production of recombinant fibrinogen as a biopharmaceutical is the achievement of safety, quality and stable supply.

To obtain recombinant fibrinogen as a biopharmaceutical, we established an expression cell line that stably produces human fibrinogen using CHO DG44 cells. We further established not only an expression system yielding more than 1.3 g/l of recombinant human fibrinogen in the culture medium by optimizing the culture conditions but also a purification process

with a yield of more than 50%, while introducing virus inactivation processes to ensure safety. The functional properties of recombinant fibrinogen obtained by us were almost equivalent to the properties of plasma fibrinogen.

We propose that the high-level expression and the effective purification process were achieved by inhibiting the degradation of recombinant fibrinogen. The observation of almost no fibrinogen degradation products in the culture medium more than 2 weeks after the start of culture showed that the culture process was not affected by the activation of proteases, which degrade fibrinogen. In addition, our adaptation of multi-mode chromatography to production without concentrating the culture supernatant or performing buffer exchange shortened the treatment time for the culture supernatant, simplified the operation and resulted in high recovery of fibrinogen.

In general, modification and denaturation of proteins can be caused by physical agents or chemical agents such as UV irradiation, organic solvents and detergents (30). Thus, we hypothesized that introducing virus-inactivation processes based on UV and SD would also reduce the quality of recombinant fibrinogen. However, there were no problems with the properties of recombinant fibrinogen produced under the conditions of virus inactivation adopted by us. Establishing conditions for the incorporation of a virus-inactivation process without reduction of quality will make a valuable contribution to development as a biopharmaceutical.

With respect to differences in the structural properties between recombinant and plasma-derived fibrinogen, there were differences between the sizes of α chains and between the added carbohydrate chains to β and γ chains, although there were no harmful influences on the function of fibrinogens. We propose that at present, there is little risk for safety because the C-terminal deletion of α chains can occur as a result of processing in nature and because the carbohydrate chains that were added to recombinant fibrinogen are presumed to be those generally added to recombinant proteins produced by CHO cells, which are typical host cells for biopharmaceuticals. However, the absence of toxicity should be confirmed in future by non-clinical safety studies.

In our preliminary study, we estimated the function of adhesion and haemostasis using this recombinant fibrinogen as a fibrin sealant mimic and obtained almost the same results as obtained using plasma fibrinogen (data not shown). We expect that the development of pharmaceuticals and devices using our recombinant fibrinogen will be possible.

Finally, further improvement of purity is required for advancing clinical development. We have already established a small-scale preparation method yielding a high-purity product and expect little difficulty in rapid establishment of recombinant fibrinogen production at the commercial level. The optimization of recombinant fibrinogen production has progressed favourably, and we will continuously improve the product quality as a biopharmaceutical for therapeutic use.

In future, we plan to develop a combination product using recombinant human fibrinogen and the recombinant human thrombin reported previously (22) for application in the field of surgical haemostatic and sealing agents and to propose its application in regenerative medicine and similar fields.

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Conflict of Interest

None declared.

References

- Martinez, J.L., Liu, L., Petranovic, D., and Nielsen, J. (2012) Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation. *Curr. Opin. Biotechnol.* **23**, 965–971
- Houdebine, L.M. (2009) Production of pharmaceutical proteins by transgenic animals. *Comp. Immunol. Microbiol. Infect. Dis.* **32**, 107–121
- Fischer, R., and Emans, N. (2000) Molecular farming of pharmaceutical proteins. *Transgenic Res.* **9**, 279–299; discussion 277
- Currie, L.J., Sharpe, J.R., and Martin, R. (2001) The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review. *Plast. Reconstr. Surg.* **108**, 1713–1726
- Spotnitz, W.D. (2014) Fibrin sealant: the only approved hemostat, sealant, and adhesive: a laboratory and clinical perspective. *ISRN Surg.* **2014**, 203943
- Mandell, S.P., and Gibran, N.S. (2014) Fibrin sealants: surgical hemostat, sealant and adhesive. *Expert Opin. Biol. Ther.* **14**, 821–830
- Bach-Gansmo, E.T., Halvorsen, S., Godal, H.C., and Skjongsberg, O.H. (1994) Impaired coagulation of fibrinogen due to digestion of the C-terminal end of the A alpha-chain by human neutrophil elastase. *Thromb. Res.* **73**, 61–68
- Holm, B., Brosstad, F., Kierulf, P., and Godal, H.C. (1985) Polymerization properties of two normally circulating fibrinogens, HMW and LMW. Evidence that the COOH-terminal end of the a-chain is of importance for fibrin polymerization. *Thromb. Res.* **39**, 595–606
- Phillips, H.M. (1981) Isolation of size homogeneous preparations of high molecular weight and low molecular weight fibrinogens. *Can. J. Biochem.* **59**, 332–342
- Laki, K. (1951) The polymerization of proteins; the action of thrombin on fibrinogen. *Arch. Biochem. Biophys.* **32**, 317–324
- Shulman, S., Katz, S., and Ferry, J.D. (1953) The conversion of fibrinogen to fibrin. XIII. Dissolution of fibrin and inhibition of clotting by various neutral salts. *J. Gen. Physiol.* **36**, 759–766
- Burnouf, T., and Padilla, A. (2006) Current strategies to prevent transmission of prions by human plasma derivatives. *Transfus. Clin. Biol.* **13**, 320–328
- Carlson, M.A., Calcaterra, J., Johanning, J.M., Pipinos, I.I., Cordes, C.M., and Velandar, W.H. (2014) A totally recombinant human fibrin sealant. *J. Surg. Res.* **187**, 334–342

14. Calcaterra, J., Van Cott, K.E., Butler, S.P., Gil, G.C., Germano, M., van Veen, H.A., Nelson, K., Forsberg, E.J., Carlson, M.A., and Velander, W.H. (2013) Recombinant human fibrinogen that produces thick fibrin fibers with increased wound adhesion and clot density. *Biomacromolecules* **14**, 169–178
15. Butler, S.P., O'Sickey, T.K., Lord, S.T., Lubon, H., Gwazdauskas, F.C., and Velander, W.H. (2004) Secretion of recombinant human fibrinogen by the murine mammary gland. *Transgenic Res.* **13**, 437–450
16. Butler, S.P., van Cott, K., Subramanian, A., Gwazduaskas, F.C., and Velander, W.H. (1997) Current progress in the production of recombinant human fibrinogen in the milk of transgenic animals. *Thromb. Haemost.* **78**, 537–542
17. Prunkard, D., Cottingham, I., Garner, I., Bruce, S., Dalrymple, M., Lasser, G., Bishop, P., and Foster, D. (1996) High-level expression of recombinant human fibrinogen in the milk of transgenic mice. *Nat. Biotechnol.* **14**, 867–871
18. Radulovic, V., Baghaei, F., Blixter, I.F., Samuelsson, S., and Jeppsson, A. (2012) Comparable effect of recombinant and plasma-derived human fibrinogen concentrate on ex vivo clot formation after cardiac surgery. *J. Thromb. Haemost.* **10**, 1696–1698
19. Gorkun, O.V., Veklich, Y.I., Weisel, J.W., and Lord, S.T. (1997) The conversion of fibrinogen to fibrin: recombinant fibrinogen typifies plasma fibrinogen. *Blood* **89**, 4407–4414
20. Lord, S.T., Binnie, C.G., Hettasch, J.M., and Strickland, E. (1993) Purification and characterization of recombinant human fibrinogen. *Blood Coagul. Fibrinolysis*. **4**, 55–59
21. Carta, G., and Jungbauer, A. (eds.). (2010) Downstream processing of biotechnology products in *Protein Chromatography*, pp. 1–55, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
22. Meta, A., Hirashima, M., Imamura, T., Kawamura, R., Yano, K., Uehara, K., and Nakashima, T. (2015) High-yield preparation of recombinant human alpha-thrombin for therapeutic use. *J. Biosci. Bioeng.* **120**, 432–437
23. Watanabe, S., Watanabe, S., Sakamoto, N., Sato, M., and Akasaka, K. (2006) Functional analysis of the sea urchin-derived arylsulfatase (Ars)-element in mammalian cells. *Genes Cells* **11**, 1009–1021
24. Akasaka, K., Nishimura, A., Takata, K., Mitsunaga, K., Mibuka, F., Ueda, H., Hirose, S., Tsutsui, K., and Shimada, H. (1999) Upstream element of the sea urchin arylsulfatase gene serves as an insulator. *Cell Mol. Biol.* **45**, 555–565
25. Yonemura, H., Imamura, T., Soejima, K., Nakahara, Y., Morikawa, W., Ushio, Y., Kamachi, Y., Nakatake, H., Sugawara, K., Nakagaki, T., and Nozaki, C. (2004) Preparation of recombinant alpha-thrombin: high-level expression of recombinant human prethrombin-2 and its activation by recombinant ecarin. *J. Biochem.* **135**, 577–582
26. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
27. Brennan, S.O. (1997) Electrospray ionisation analysis of human fibrinogen. *Thromb. Haemost.* **78**, 1055–1058
28. Adamczyk, B., Struwe, W.B., Ercan, A., Nigrovic, P.A., and Rudd, P.M. (2013) Characterization of fibrinogen glycosylation and its importance for serum/plasma N-glycome analysis. *J. Proteome Res.* **12**, 444–454
29. Hossler, P., Khattak, S.F., and Li, Z.J. (2009) Optimal and consistent protein glycosylation in mammalian cell culture. *Glycobiology* **19**, 936–949
30. Puri, D. (2011) Amino acids peptides and proteins in *Textbook of Medical Biochemistry*, 3rd edn, pp. 47–74, Elsevier India, Gurgaon