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γ375W fibrinogen-synthesizing CHO cells indicate the accumulation of variant fibrinogen within endoplasmic reticulum

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Abstract

Background: Hepatic endoplasmic reticulum (ER) storage disease (HERSD) associated with hypofibrinogenemia has been reported in patients with four types of heterozygous γ-chain variant fibrinogen in the C terminal region. Of interest, substitution of γR375W induced hypofibrinogenemia and HERSD, whereas γR375G induced dysfibrinogenemia. *Objectives:* To analyze the synthesis of variant fibrinogen and morphological characteristics, we established variant fibrinogen-producing cells and compared them with wild-type fibrinogen-synthesizing cells. *Methods:* The fibrinogen γ-chain expression vectors coding γ375W and γ375G were altered by oligonucleotide-directed mutagenesis and transfected into Chinese hamster ovary (CHO) cells. Synthesis of fibrinogen (media and cell lysates) was measured by ELISA for each cloned cell line and morphological characteristics were observed by immunofluorescence and transmission electron microscopy. *Results:* The medium/cell lysate fibrinogen ratio of γ375W-CHO cells was markedly lower than that of the normal cells and γ375G-CHO cells. Immunostaining with anti-fibrinogen antibody showed only γ375W-CHO cells, but revealed two types of cells containing cytoplasmic inclusion bodies, scattered large-granular bodies and fibrous forms. Observation by confocal microscopy indicated that both inclusion bodies were colocalized with fibrinogen and ER-membrane protein; furthermore, transmission electron microscopic observation demonstrated dilatation of the ER by large-granular inclusion bodies and fibrous forms filled with regularly

structured fibular materials within the dilated ER. *Conclusion:* These results demonstrated that assembled and non-secreted γ375W fibrinogen was accumulated in the dilated ER and aggregated variant fibrinogen was seen as regularly structured fibular materials, which was similar to the fingerprint-like pattern observed at inclusion bodies in patients' hepatocytes affected with HERSD.

Keywords: endoplasmic reticulum, fibrinogen storage disease, fibrinogen γ-chain mutation, hepatic endoplasmic reticulum storage disease, hypofibrinogenemia.

Introduction

Fibrinogen is a 340-kDa plasma glycoprotein synthesized in hepatocytes and secreted into the blood, and plays crucial roles in blood clotting, wound healing, inflammation, angiogenesis, and cellular and matrix interactions [1]. Circulated fibrinogen consists of two copies of three polypeptide chains, Aα, Bβ, and γ, linked by an extensive network of 29 intra- and inter-chain disulfide bonds [2,3]. After synthesis in hepatocytes, individual polypeptide chains are translocated into the rough endoplasmic reticulum (ER) and initially Aα-γ and Bβ-γ intermediates are formed with disulfide bonds and subsequently connected with Bβ- or Aα-chain, leading to the formation of a half molecule (Aα- Bβ-γ) [4,5]. Two half molecules are assembled into a hexameric molecule with disulfide bonds, folded, and glycosylated in the ER and Golgi apparatus

before secretion. Aberrantly conformed fibrinogen or unassembled individual polypeptide chains are retained within the ER and degraded by the proteasome pathway as the ER-associated protein degradation (ERAD) system [6,7].

Hypofibrinogenemia or afibrinogenemia, defined as reduced or negligible levels of fibrinogen in plasma, can be hereditary. Genetic abnormalities in patients with these diseases have been found in all three genes and identified as missense, nonsense, or frameshift mutations; splice-site abnormalities; or large deletions, as listed in the fibrinogen variant database [updated on 26/01/2012, http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html]. Some patients with only four variant types of hypofibrinogenemia, substitution of γG284R[8], γT314P [9], and γR375W mutation [10-14], and deletion of γG346-Q350 [15] (all numbered as mature protein without signal peptide) have been reported to show onset of liver disease in so-called hepatic ER storage disease (HERSD) [16] or fibrinogen storage disease, being in the same category as genetic deficiency of α-1-antitrypsin (A1AT) (G342K, Z-mutation) [17].

We have already established eight variant fibrinogen-synthesizing CHO cells, all showing reduced secretion of variant fibrinogen into the media [18,19]. In addition, these cells showed elevated levels of cytoplasmic fibrinogen. Namely, some single

amino acid substitutions of the γ-chain C-terminal region induced normal assembly, reduced secretion, and accumulation of fibrinogen molecules within the cells. However, hypofibrinogenemic patients affected with six of eight variants of fibrinogen (another two variants have not been found to be hypofibrinogenemia) have not been reported to show the onset of liver diseases.

In this study, to analyze the variant fibrinogen-producing Chinese hamster ovary (CHO) cells causing HERSD, we established CHO cells synthesizing γ375W, γ375G, or γ375E fibrinogen, and compared the synthesis of fibrinogen and morphological characteristics because the variant fibrinogen, γR375W, was found in HERSD patients with heterozygous hypofibrinogenemia [10-14] and fibrinogen Osaka V, γR375G, was reported as heterozygous dysfibrinogenemia [20]. Immunofluorescence using anti-fibrinogen antibody showed that only γ375W, not γ375G or γ375E fibrinogen-synthesizing CHO cells, formed cytoplasmic inclusion bodies. To characterize the inclusion bodies we observed the γ375W fibrinogen-synthesizing CHO cells by confocal laser scanning microscopy (CLSM) and transmission electron microscopy.

Materials and methods

Construction of mutant expression vectors and recombinant protein expression.

The fibrinogen γ chain expression vector pMLP-γ (γN) was altered by oligonucleotide-directed mutagenesis using the QuikChange II Site-Directed Mutagenic Kit (Stratagene, La Jolla, CA, USA) and the following primer pairs (the altered base is underlined); 5'-GCC ACT TGG AAA ACC TGG TGG TAT TCC ATG-3'and 5'-CAT GGA ATA CCA CCA GGT TTT CCA AGT GGC-3' for γ375W, 5'-GCC ACT TGG AAA ACC GGG TGG TAT TCC ATG-3' and 5'-CAT GGA ATA CCA CCC GGT TTT CCA AGT GGC-3' for γ375G, and 5'-GCC ACT TGG AAA ACC GAG TGG TAT TCC ATG-3' and 5'-CAT GGA ATA CCA CTC GGT TTT CCA AGT GGC-3' for γ375E according to the instruction manuals. To confirm the insertion of each mutation, the complete γ-chain cDNAs of the plasmids were sequenced as described previously [21].

The resultant expression vectors, γ375W, γ375G, γ375E, and γ chain expression vector (γ375R) were transfected into CHO cells that expressed normal human fibrinogen Aα and Bβ chains (AαBβ-CHO cells) using the standard calcium-phosphate co-precipitation method, and stable transfectants were selected using both G418 (GIBCO BRL, Rockville, MD, USA) and histidinol (Aldrich Chemical, Milwaukee, WI, USA) and the cell lines were designated as γ375W-, γ375G-, γ375E-, and γ375R-CHO cells, respectively. Nine to twelve colonies from fibrinogen-synthesizing CHO cells were selected at random, expanded, and examined for fibrinogen synthesis. The cells

were cultured in Dulbecco's modified Eagle's medium Ham nutrient mixture F12, supplemented as described (DMEM-F12 medium).

Enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis

Fibrinogen concentrations in culture media or cell lysates were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [21]. Culture medium for immunological analysis was prepared as follows. Cells were grown to confluence in 60-mm dishes (approximately 1.5×10^6 - 2.0×10^6 cells), and the conditioned medium was harvested 1 day after reaching confluence (6-8 days after seeding) for ELISA. Cells were harvested from the same culture dishes, washed 3 times, and finally lysed with 250 μL of 0.1% IGEPAL CA-630 (Sigma, St Louis, MO, USA) and 10 mM phenylmethylsulfonyl fluoride (Sigma) in 50 mM Tris-HCl buffer pH 8.0.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis were performed as described previously [21]. Briefly, immunoblots were developed with a rabbit anti-human fibrinogen antibody (Dako, Carpinteria, CA, USA) and the reacting species were visualized using horseradish peroxidase conjugate-goat anti-rabbit IgG antibody (Medical and Biological Laboratories, Nagoya, Japan) and enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were exposed on

Hyperfilm-ECL (Amersham Pharmacia Biotech).

Statistical analysis

The statistical significance of differences between the normal control and variant fibrinogen-producing cells was determined using unpaired t tests. A difference was considered significant when $p<0.05$.

Immunofluorescence staining

For immunofluorescence microscopy, the cells grown to 80% confluent on cover-glass in 60-mm culture dish were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Localization of intracellular fibrinogen was identified by incubating for 1h with 1:50 diluted FITC-labeled sheep polyclonal anti-fibrinogen antibody (The Binding Site Group Ltd., Birmingham, UK) in 1% bovine serum albumin containing phosphate-buffered saline (BSA/PBS). Fluorescence was observed with a fluorescence microscope (BX60; OLYMPUS, Tokyo, Japan).

For double immunofluorescence microscopy, fixation and permeabilization of the cells were performed as described above. In staining for an ER membrane marker, CHO cells were incubated with 1:50 diluted rabbit polyclonal anti-calnexin antibody (Abcam, Cambridge, UK) for 1h, followed by 1:500 diluted Texas Red-labeled goat anti-rabbit

IgG secondary antibody (Abcam, Cambridge, UK) for 1h. In staining for fibrinogen, CHO cells were incubated with FITC-labeled sheep polyclonal anti-fibrinogen antibody for 1h simultaneously with Texas Red-labeled secondary antibody. Stained samples were mounted with VECTASHIELD Hard-Set Mounting Medium (Funakoshi, Tokyo, Japan) and observed with a confocal laser scanning microscope (LSM 510 META; Zeiss, Oberkochen, Germany). Samples were observed using 0.8 μm or 0.4 μm optical sections.

Transmission electron microscopy

For transmission electron microscopy, the cells grown to 80% confluence on cover-glass in a 60-mm culture dish were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4 °C overnight, followed with 1% osmium tetroxide in 0.1M phosphate buffer at 4 °C 30 min. After dehydration, cells were embedded in epoxy resin. Ultrathin sections (90 nm) were placed in 200 mesh copper grids and stained with uranyl acetate (10 min) and lead citrate (5 min) and coated with a thin carbon layer. The specimens were observed on JEM -1230 (JEOL, Tokyo, Japan) and images were recorded at 30000 × with an 80 kV accelerating voltage. ER diameters were measured on a 267% enlargement from an image of 30 000× magnification.

Results

Synthesis and secretion of recombinant variant fibrinogens in CHO cells

The variant fibrinogen or normal fibrinogen was expressed in CHO cells and 9 to 12 fibrinogen-synthesizing cell lines each were selected as described in Materials and Methods. The fibrinogen concentrations in the cell lysates and culture media were measured by ELISA. The results are shown in Fig. 1. The fibrinogen concentrations in γ375R-CHO cells (mean ± SD, n = 11) were 1.07 ± 0.09 μg/L for the culture media and 1.16 ± 0.16 μg/L for the cell lysates, resulting in a ratio of culture media to cell lysates of 0.94 ± 0.10. The concentrations in the cell lysates of each of the variant fibrinogen-expressing cell lines were significantly elevated compared with in normal cells; γ375G-CHO cells, 1.5-fold; γ375E-CHO cells, 2.0-fold; γ375W-CHO cells, 3.3-fold (Fig. 1-B). Moreover, the concentrations in the culture media of γ375G- and γ375E-CHO cells were significantly elevated compared with that in normal cells, 1.8- and 2.1-fold, respectively. On the other hand, the concentration in the culture media of γ375W-CHO cells was significantly lower than that in the normal cells (0.32-fold) (Fig. 1-A). The medium/cell lysate fibrinogen ratio of γ375W-CHO cells was markedly lower (0.09 ± 0.02) than that of the normal cells (0.94 ± 0.10) and other two mutant cells (γ375G-CHO cells; 1.10 ± 0.25, γ375E-CHO cells; 1.00 ± 0.53, respectively (Fig. 1-C).

To observe the assembly of intact fibrinogen and/or synthesis of a mutant γ-chain,

SDS-PAGE and immunoblotting analysis were performed under non-reducing or reducing conditions. Under non-reducing conditions, intact fibrinogen was observed in cell lysates of all three fibrinogen variants, including γ375W and normal fibrinogen-expressing cell lines (Fig. 2-A). Under reducing conditions, not only Aα- and Bβ-chain but also γ-chain were observed in cell lysates of three variant fibrinogen and normal fibrinogen-expressing cell lines (Fig. 2-B). Interestingly, the amount of the γ-chain band of γ375W-CHO cells was higher than that of other fibrinogen-expressing cell lines.

Fibrinogen staining for variant fibrinogen-synthesizing CHO cells

To analyze the localization of variant fibrinogen in established CHO cells, a direct immunofluorescence test with FITC labeled anti-fibrinogen antibody was performed as described in Materials and Methods. Original CHO cells (Fig. 3A) did not produce fibrinogen and were used as a negative control. Normal fibrinogen-synthesizing γ375R-CHO cells were stained in a perinuclear network pattern (Fig.3B) and used as a positive control. Variant fibrinogen-synthesizing cell lines, γ375G-CHO cells (Fig. 3C) and γ375E-CHO cells (Fig. 3D) were stained in a similar pattern to γ375R-CHO cells. Surprisingly, γ375W-CHO cells (Fig. 3-E, F) showed three staining patterns, namely scattered large-granular bodies (abbreviated as ‘g’), fibrous forms of two subpolar sites

of nucleus (abbreviated as ‘f’), and a perinuclear network (abbreviated as ‘n’). On day 3 after subculture, the frequency of each staining pattern (‘g’, ‘f’ and ‘n’) was 32.6%, 22.4%, and 44.9%, respectively. However, CHO cells having two aberrant patterns in a single cell, namely ‘g’ + ‘f’ pattern, were seen rarely (<0.5%).

Analyses of aberrant structure of γ375W fibrinogen-synthesizing CHO cells.

To analyze where γ375W-fibrinogen was accumulated and from where the aberrant structure of γ375W fibrinogen-synthesizing CHO cells was derived, observation using confocal laser scanning microscopy (CLSM) was performed for stained cells with anti-calnexin antibody (calnexin is an ER-membrane marker) (red) and anti-fibrinogen antibody (green), as described in Materials and Methods. As shown by confocal microscopy, ER-membrane protein was localized typically in a perinuclear network pattern. In addition, the aberrant structure of scattered large-granular bodies (Fig. 4 C, D) and fibrous forms (Fig. 4 C, E) in γ375W-CHO cells co-localized with calnexin, resulting in a yellow merged signal (Fig. 4 C-E).

To ascertain the accumulation of γ375W-fibrinogen in aberrant structured ER, we observed the ER in CHO cells by transmission electron microscopy. Images are shown in Fig. 5. ER width of γ375R-CHO cells as a normal control (Fig. 5A, D) were 64.0 ± 13.6 nm (n = 14). On the other hand, ER of γ375W-CHO cells with scattered large

granular bodies (Fig. 5B, E) showed enlarged vacuoles with diameters of 760.4 ± 125.5 nm (n = 6). For γ375W-CHO cells with fibrous forms (Fig. 5C, F), the width of the ER was expanded slightly, 176.6 ± 34.7 nm (n = 22) and, moreover, the ER lumen was filled with regularly structured fibular materials. These appeared to have a fingerprint-like pattern, as shown in hepatocytic inclusion bodies of HERSD patients by transmission electron microscopy [10-14].

Changes of aberrant structure of γ375W fibrinogen-synthesizing CHO cells and subcloning.

To analyze whether two types of aberrant cytoplasmic inclusion in γ375W-CHO cells, large granular bodies (‘g’) and fibrous forms (‘f’), are the same clone and derived from the normal type as a perinuclear network (‘n’) or a transition of each other, we stained CHO cells by immunofluorescence from day 1 to day 7 after subculturing (Fig. 6). On day 1, the frequency of each staining pattern (‘g’, ‘f’, and ‘n’) was 23.5, 10.1, and 66.4%, ‘n’ pattern cells decreased and ‘g’ and ‘f’ pattern cells increased according to the culture period, and finally, on day 7 ‘g’, ‘f’, and ‘n’ pattern cells were 47.7, 50.0, and 2.3%, respectively. In addition to the frequency change of the staining pattern, the intracellular fibrils elongated each day (Fig. 6). In addition, double aberrant patterns of ‘g’ and ‘f’ were seen in CHO cells on day 3 (Fig. 6C).

Furthermore, we performed subcloning of γ375W fibrinogen-synthesizing CHO cells and the yielded clones comprised cells showing only the ‘n’ pattern or mixed cells showing the ‘g’ and ‘f’ pattern. No clones with only the ‘g’ or ‘f’ pattern were yielded (data not shown).

Discussion

To examine the assembly and/or secretion of variant fibrinogens substituted at γR375 residues, γ375G, γ375E, and γ375W were expressed in CHO cells as stable transfectants. All these cell lines synthesized a mutant γ-chain, and assembled it into fibrinogen in the cells. Moreover, the former two variants secreted a large amount of fibrinogen into the culture media but the latter secreted a markedly small amount. Variant fibrinogen Osaka V, γR375G, was reported as heterozygous dysfibrinogenemia; namely, the plasma fibrinogen concentration determined by the thrombin time method was 0.9 g/L and that determined by an immunological method was 2.5 g/L [20]. On the other hand, HERSD-induced variant known as fibrinogen Aguadilla; γR375W, was reported as heterozygous hypofibrinogenemia; namely, the plasma fibrinogen concentration determined by the thrombin time method and immunological method was 0.6 g/L by both [10]. Another four identical variant fibrinogens also reported lower levels of plasma fibrinogen and were diagnosed as hypofibrinogenemia [11-14]. Overall,

considering the excretion pattern of γ375W we conclude that our recombinant fibrinogen-producing system using CHO cells gives results similar to patients with hypofibrinogenemia.

To analyze the accumulation and localization of variant fibrinogen in the cytoplasm of γR375 variant CHO cells, immunofluorescence staining was performed. Surprisingly, only γ375W-CHO cells showed two types of cytoplasmic inclusion bodies, scattered large granular bodies and fibrous forms. Observation by confocal microscopy demonstrated that these aberrant structures were colocalized with ER-membrane protein. Further observation by transmission electron microscopy indicated dilatation of the ER by large granular bodies and filling with regularly structured fibular materials within the ER on a fibrous structure. These results demonstrated that assembled and non-secreted γ375W fibrinogen was accumulated in the dilated ER and aggregated variant fibrinogen was seen as regularly structured fibular material, being similar to the fingerprint-like pattern observed at inclusion bodies in patients' hepatocytes affected with HERSD [10-14]. In addition to γR375W mutation, γG284R[8], γT314P [9], and deletion of γG346-Q350 [15], all mutations residing in the globular γD domain, have been reported as HERSD with hypofibrinogenemia, and all also demonstrated the presence of hepatocellular cytoplasmic inclusion bodies filled with anti-fibrinogen antibody-reacting materials and were also observed as tubular materials with a

fingerprint-like pattern by electron microscopy [8,9,15]. ER accumulation of variant protein inducing liver cirrhosis (HERSD) was originally reported in a deficiency (homozygotes) of A1AT (G342K, Z-mutation) [17]. In general, it is well known that improperly folded proteins and aggregated proteins are often retained and/or degraded within the ER [22]. In addition to A1AT and fibrinogen, variants of other acute phase proteins (α-1-antichymotrypsine, antiplasmin, C3, and C4) and defects of low-density lipoprotein (LDL) receptor have been shown to be retained within the ER [16].

Kruse *et al.* demonstrated using a yeast expression system that variant γD domain of γ375W was cleared from the ER via ER-associated protein degradation, and when the ER was saturated, aggregated variant γD domain was accumulated within the ER and sent to vacuoles for degradation via the autophagy pathway [23]. In brief, the report suggested the possibility that HERSD associated with variant fibrinogenemia might be caused by decreasing function of the autophagic pathway. Moreover, molecular mechanisms of A1AT homozygous Z mutation causing hepatocyte injury have been studied extensively and showed increased ER retention as a conformational disease [24], activation of ER-specific stress responses [25], up-regulated mitochondrial autophagy [26], or mitochondrial injury and apoptosis [27]. Our γ375W-CHO cells indicated cytoplasmic aberrant inclusion bodies with large granular or fibrous forms and this suggested that variant fibrinogen was retained and aggregated within the dilated ER. In

this report, we have not elucidated the molecular mechanism of the formation of cytoplasmic aberrant inclusion bodies in γ375W-CHO cells; however, there is a possibility that the degree of fibrinogen accumulation, secretion, and/or degradation might be different in species and/or organ (cell lines). Therefore, we have to establish human hepatocyte cell lines (HL-7702 and HepG2 cells etc.), perform immunofluorescence staining, and analyze and compare the molecular mechanism of the formation of cytoplasmic inclusion bodies.

In conclusion, γ375W-CHO cells indicated that the assembly of variant fibrinogen was normal but secretion was markedly reduced and these observations suggest that hypofibrinogenemia is induced in heterozygous γR375W patients. In addition, cytoplasmic aberrant inclusion bodies in γ375W-CHO cells, especially fibrous forms, were comparable to the hepatocellular inclusion bodies observed in hypofibrinogenemia patients associated with HERSD. We hope that immunofluorescence staining of CHO cells and/or liver cells accumulating variant fibrinogens will be used as a screening procedure for possible HERSD before onset (elevated liver enzyme in serum). Moreover, these cells are useful for to analyzing the molecular mechanisms of ER storage disease and conformational disease causing inclusion bodies to accumulate unfolded protein within the cells or matrix.

Authorship

Study concept and design, drafting of the manuscript: N. Okumura, F. Terasawa, T. Kobayashi. Experiment: T. Kobayashi, S. Arai, N. Ogiwara, Y. Takezawa, M. Nanya. Data analysis and figure preparation: T. Kobayashi, M. Nanya. Critical revision of the manuscript: S. Arai, N. Ogiwara, Y. Takezawa.

Conflicts of Interest

None of the authors have any conflicts of interest with regards to this work.

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Figure and legends

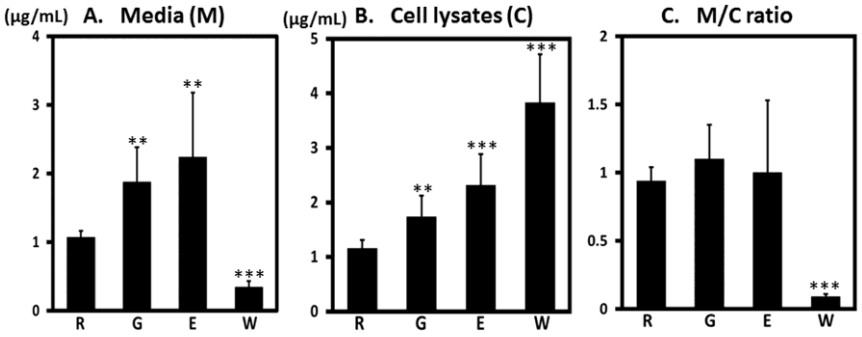

Figure 1. *Synthesis of variant fibrinogens in transfected CHO cells.*

The concentrations of fibrinogen in the culture media (A) and cell lysates (B) were measured by ELISA, as described in "Materials and methods." Fibrinogen concentration ratios of medium to cell lysate are shown in panel C. The mean values are presented with standard deviations, indicated by error bars. Concentrations were determined for 9 to 12 isolates of CHO cell lines expressing γ375R (wild type), γ375G, γ375E and γ375W. Significantly different from γ375R: (*P <0 .05, **P <0 .01, ***P <0 .001). R:γ375R-CHO cells (n = 11), G: γ375G-CHO cells (n = 9), E:γ375E-CHO cells (n = 9), W: γ375W-CHO cells (n = 12).

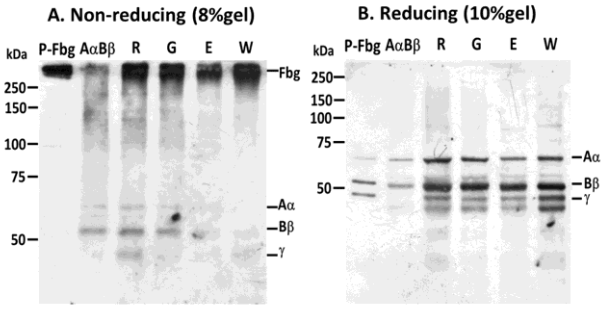

Figure 2. *Western blot analysis of CHO cell lysates and culture media.*

Samples of CHO cell lysate were subjected to 8% SDS-PAGE under non-reducing conditions (A) or 10% SDS-PAGE under non-reducing conditions (B). After transfer to a nitrocellulose membrane, blots were developed as described in "Materials and Methods". P-Fbg: purified fibrinogen from normal control plasma, AαBβ: Aα- and Bβ chain-producing CHO cells, R: γ375R-CHO cells, G: γ375G-CHO cells, E: γ375E-CHO cells, W: γ375W-CHO cells.

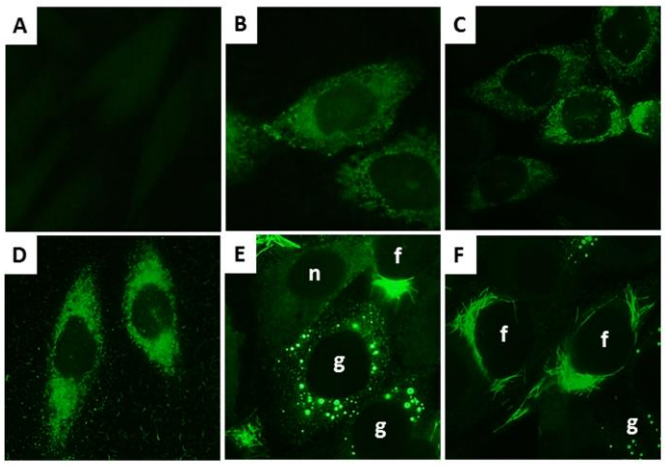

Figure 3. *Immunofluorescence staining for variant fibrinogen-synthesizing CHO cells.* Fixed and permeabilized CHO cell lines were stained with FITC-labeled anti-fibrinogen antibody, as described in "Materials and Methods". CHO cells (A) as a negative control, γ375R-CHO cells (B) as a positive control, γ375G- (C), γ375E- (D), and γ375W-CHO cells (E, F). γ375W shows two aberrant and a normal form; g: scattered large granular bodies, f: fibrous forms, and n: perinuclear network. Original magnification: 1000×.

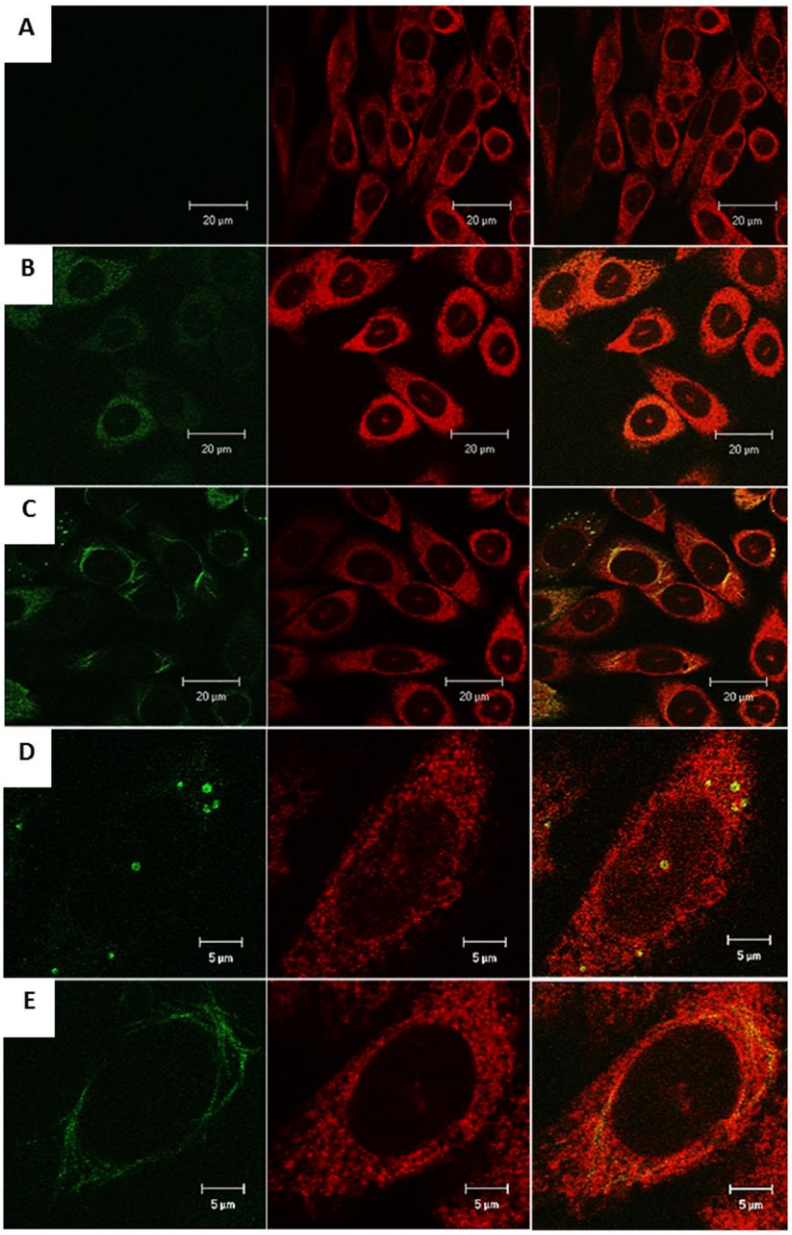

Figure 4. *Double immunofluorescence staining and observation using confocal laser scanning microscopy.*

CHO cells were stained with FITC labeled anti-fibrinogen antibody (left and green) and anti-calnexin antibody plus Texas Red-labeled second antibody (middle and red), and merged images (right and yellow), as described in “Materials and Methods”. CHO cells (A) as a negative control, γ375R-CHO cells (B) as a positive control, γ375W-CHO cells (C-E). Scattered large granular bodies (D) and fibrous forms (E) of γ375W-CHO cells. Optical section: 0.8μm (A-C), 0.4μm (D, E), scale bar: 20μm (A-C), 5μm (D, E). Original magnification: 1000× (A-C). Optical magnification: 3000× (D, E).

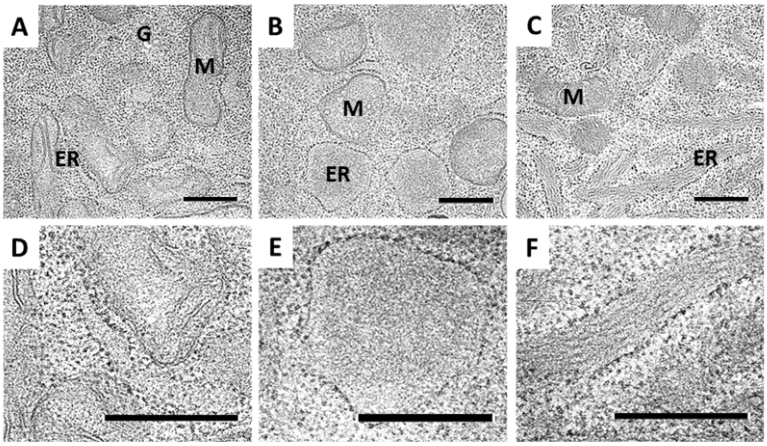

Figure 5 *Ultrastructure of ER in γ375W-CHO cells.*

Ultrastructure of ER in CHO cells was observed by transmission electron microscopy. γ375R-CHO cells (A,D) as a normal control, γ375W-CHO cells with scattered large granular bodies (B,E), γ375W-CHO cells with fibrous forms (C, F). M: mitochondrion, G: Golgi apparatus, ER: endoplasmic reticulum. Scale bar: 0.5μm (A-F).

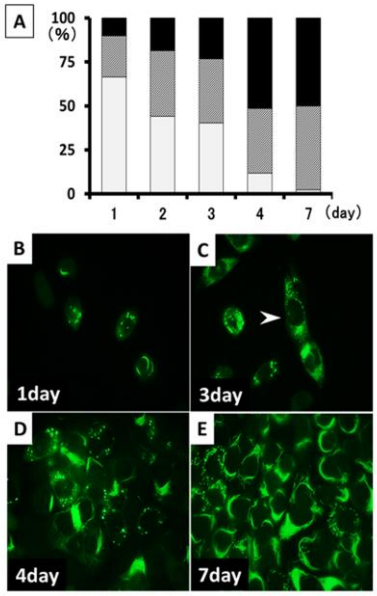

Figure 6 *Changes in aberrant structure of γ375W fibrinogen-synthesizing CHO cells.*

Changes of staining pattern between day 1 and day 7 after subculture are shown in panel A (black bars: large granular bodies, hatched bars: fibrous forms, and white bars: perinuclear network). Representative staining is shown in panel B to E.