國科會專題研究計劃之研究成果報告

計劃名稱：血友病第八因子的生合成及其細胞內運轉過程的研究 (3/3)

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Progress Reports

Abstract

Most evidence to date indicates that the vWF has either a stabilizing effect, or that the vWF can elicit the release of FVIII from storage depots or stimulate the synthesis or secrection of FVIII (1). Moreover, in Xie's group, they found the multimer size of vWF produced endogenously by HUVEC was similarly reduced following secretion in the conditioned medium (2). Therefore, we decided to express FVIII in human kidney 293 cells under the modified cultured system. In this system, you used HUVECs, which can naturally release vWF that can protect FVIII from degradation, as feeder layers or directly feed HUVEC growth medium for culturing 293 cells rather than co-express vWF (3) to avoid the disruption of the secretion of FVIII in order to increase the expression level of FVIII and maintain the stability of FVIII. In order to understand the intracellular fate of FVIII, wild type FVIII without any deletion, and mutants with single base substitutions at different position based on the two hemophilia A patients (Table 1), were expressed in human kidney 293 cells under the above modified cultured system. The two monoclonal antibodies, D2 and E6, produced in our laboratory have been used in our experiments. The epitopes for D2 was identified to the thrombin cleavage site at amino acid residue1689 of FVIII, and E6, within the B domain at residues 712-1648 of FVIII. FVIII has been known to accumulate in the ER and Golgi apparatus in the normal liver tissues. Surprisingly, our results showed that D2 can detect strong signals in the nucleus, and E6, in the mitochondria from electronic microscopy. Although the explanation for this finding is not known at the present time, we have attempted to co-localize FVIII with 2 heat shock proteins, hsp60 and mitochondrial hsp70 (mtHsp70). Our results have demonstrated, for the first time, that FVIII might be at different localization in the different organelle during the secretion. Moreover, FVIII may exist the different protein form in the mitochondria and nucleus compared with FVIII in the plasma.

Introduction

Anti-hemophilic factor VIII (FVIII) is a 330-kDa glycoprotein essential for blood clotting at the damaged vessel wall (4,5). Deficiency of FVIII, whether at the transcriptional or the translational level, causes an X-linked inherited bleeding disease, hemophilia A (6). About one in five to ten thousand males suffers this disorder worldwide (6,7).

The gene coding for FVIII was cloned in 1984 (8). It is about 186 kb, consisting of 26 exons encoding a mRNA of 9 kb and a single chain polypeptide of 2351 amino acids (8-12).

There are several tissues proposed to be the sites for FVIII synthesis. However, hepatocyte is the most physiologically important tissue (13,14). Mature FVIII contains 2332 amino acids with a domain structure of A1-A2-B-A3-C1-C2 (from N- to C-terminus) (9,10,12,15). A1, A2 and A3 are highly homologous to one another (9,12) and to ceruloplasmin (9,12) and factor V (FV) (15), also a clotting factor.

The FVIII protein, circulating in a noncovalent complex with von Willebrand factor (vWF) (15), functions as a crucial cofactor of factor IX (4,5). FVIII is activated by thrombin to contain a heterotrimeric structure of A1, A2, and A3-C1-C2 (9,10,12,16-19). Upon activation, FVIII dissociates from vWF (19) and accelerates the activation of factor X by activated factor IX in the presence of calcium and phospholipid (4).

According to previous investigations, FVIII's light chain (A3-C1-C2) contains sites for binding of vWF (12,20-24), activated protein C (25) and negatively charged phospholipid (12,26). FVIII interacts with activated factor IX at two regions in A2 (27) and A3 domains (28,29). The B domain appears to be dispensable for FVIII function (12,30,31).

The average concentration of FVIII protein in plasma is 0.1μg/ml (4,5). Compared to FV, FVIII is almost one hundred folds lower in concentration. Hemophilia A's severity, relying upon the level of FVIII activity in blood, is classified as either mild (5-30%), moderate (1-5%) or severe (<1%) (32).

There are two therapeutic cures of hemophilia A: 1.) Infusion of FVIII concentrates derived from human plasma or recombinant sources, which may result in formation of autoantibody inhibitors in five to fifteen percent of hemophilia A patients (33); 2.) Gene therapy is limited by the low expression level of the FVIII protein and the ineffective delivery methods (33-36).

The cellular biosynthesis pathway of FVIII after translation is important for studying the cell expression of FVIII. It is yet a mystery why FVIII secretes inefficiently. Most of the FVIII proteins do not secret from the endoplasmic reticulum (ER) (19). It is now understood that FVIII and FV have different interactions with various ER-specific chaperones, such as immunoglobulin binding protein (BiP, also GRP78), calnexin, and calreticulin, in some aspects:

a. BiP binding with FVIII was proved (19) and mutagenesis of a potential BiP binding site in the A1 domain of FVIII was found to enhance secretion of FVIII (37). However, such binding with FV has never been found. The F309S mutant, in which serine is the native residue at this site in FV, resulted in the increased secretion by 2.3 fold correlated to the reduction of BiP binding (38).

b. Calnexin and calreticulin are specific for some glycoproteins (39-41). Although FVIII and FV are both heavily glycosylated proteins (5,10,42), association of calnexin could be detected only in FVIII but not in FV (43).

Calreticulin interacts with both FVIII and FV (43). BiP involves only in the binding between calreticulin and FVIII. Evidence showed that the calnexin and calreticulin binding was mostly mediated by B domain in FVIII. A B-domain-deleted-FVIII secreted ten folds higher than the wildtype FVIII, indicating an incredible relation between B domain and secretion efficiency.

c. Calnexin is believed to play a key role in the degradation of proteins that cannot fold or assemble properly in the ER (44,45). This machinery is related to the cytosolic proteasome degradation. Recently, FVIII degradation within the cells was found inhibited by the inhibitor of cytosolic 20S proteasome pathway (43). That showed some connection among chaperone interactions, cellular degradation , and the low expression level of FVIII.

Besides the chaperones, most post-translational modifications of the protein are also important to FVIII biosynthesis. N-linked glycosylation in 25 potential sites is needed for FVIII not being trapped in the cells (46). Sulfation of six tyrosine residues is required for full FVIII procoagulant activity and affects the affinity with vWF (47,48), which stabilizes FVIII and increases its half-life five times longer in plasma (12,19,49).

There are no known established or primary cell lines that express FVIII (13). Therefore, the model of FVIII destiny has been roughly proposed. After the polypeptide chain of FVIII enters the ER lumen cotranslationally (19), glycosylation is proceeding during the translocation. BiP which is associated with the membrane bound translocon binds to the polypeptide chain and together forms a complex with calreticulin (43). The complex transfers FVIII to the calnexin. Calnexin facilitates the correct folding of the polypeptide chain and maybe transport it out of the ER toward the cytosolic degradation. A small portion of the polypeptide chain within the ER is left and continues the journey for the formation of a mature protein (19). There may be other chaperones in the ER involved but it is under identification. When the protein travels to the Golgi, glycosylation, sulfation, and other modifications will be completed. It will be cleaved to heavy chain (A1-A2-B) and light chain (A3-C1-C2) before secreted from the cell (9,10,12).

Previously, we have identified the molecular defects in Chinese patients with hemophilia A (50). To understand the pathogenic mechanism of the mutations, some of the mutations are selected for further analysis. The patients with mutations at residue 146 or 386 have decreased levels of their activity and antigen level in plasma, both less than 1%, and they are in a severe phenotype. To seek the reasons for this consequence, I first focus on the cellular retention. With a technique of confocal microscopy which is already set up in our laboratory, the intracellularly differences between wildtype and mutants will be observed and discussed.

Methods

I. HUVEC isolation and culture

ECs were isolated from human umbilical vein by collagenase (0.02%) digestion and were grown to confluence on a plastic dish in medium M199 containing 10% fetal bovine serum, 10 U/ml heparin and 25 μg/ml endothelical cell growth supplement. The EC monolayer was trypsinized and resuspended in M199, and thereafter seeded on a 0.2% collagen gel-coated cover glass. The first passage cells reached confluence within 2 days and were subsequentlyused between 3 to 4 days.

II. Purification of mitochondria fractions by continuous sucrose gradient

Mouse liver homogenate was prepared in the ice-cold medium (0.25M sucrose, 1mM EDTA. 10mM Tris-Cl pH 7.4). Nuclei were pelleted by centrifuging 5 min at 1000xg 4℃ in a swinging-bucket rotor using a low speed centrifuge. Then, mitochondria were pelleted from the postnuclear supernatant by centrifuging 10 min at 15,000xg 4℃ in a fix-angle rotor using high-speed centrifuge. The mitochondria pellet was resuspended in 8 ml ice-ccold medium using 3 to 4 gentle strokes of the Dounce homogenizer. Seven to eight miniliter sample was layered on the top of the sucrose gradients (34% to 64%) and centrifuged 6 min at 170,000xg 4℃. The gadients in 1 ml fractions were collected, then samples were dilluted with $\geq$ 2 vol ice-cold medium and centrifuged 20 min at 20,000xg 4℃. Finally, pellets were resuspended in ice-cold medium with suitable volume for further experiments.

III. Succinate dehydrogenase assay for mitochondria.

Three hundred microliters succinate solution (10mM sodium succinic acid hexahydrate in phosphate buffer pH 7.4) were placed in 1.5ml microcentrifuge tubes. Ten to twenty microliters of each gradient fraction were added, then samples were incubated at 37℃. One hundred microliters INT solution (2.5 mg/ml *p*-iodonitrotetrazolium violet in phosphate buffer pH 7.4) were added and incubated for another 10 to 20 min at 37℃. The reaction was terminated by adding 1 ml stop soln I (10 g trichloroacetic acid, 50 ml ethyl acetate and 50 ml ethanol). Any precipitate was removed by microcentrifuging for 2 min at maximum speed. Finally, using glass cuvettes, the absorbance at 490 nm was measured against suitable blank.

Result

I. Built up the modified culturing system for expressing high-level full-length FVIII.

Our laboratory will use the human HepG-2, Hun7 and 293 cells to build up the expression system for wild type FVIII and mutant FVIIIs by point mutation based on the hemophilia A patients in the mammalian cells under the modified cultured system. In this system, you used HUVECs as feeder layers or directly feed HUVEC conditioned medium for culturing cells in order to increase the expression level of FVIII and

maintain the stability of FVIII. The results shown in Table 2 indicated that HUVECs may release vWF in the conditioned medium. This system was used to express full length of FVIII with the good expression level in the human cell line. In our preliminary results, the clotting time of FVIII deficient plasma with expressed FVIII under the HUVECs conditioned medium decreased indicates the expression level of FVIII is higher than that under normal medium (data not shown).

II. Determination of the existence for FVIII in mitochondria of mouse and human liver.

Two monoclonal antibodies, D2 and E6, with different reorganization epitopes have been produced in our laboratory. Surprisingly, our results previously showed that E6 could detect strong signals in the mitochondria, but not E6 in normal human liver from electronic microscopy. These results demonstrated, for the first time, that FVIII might be located in the different organelle during the secretion and may exist the different protein form in the different organelle. In our preliminary results, we tried to separate the mitochondria from the mouse liver by subcellular fractionation method. The mitochondria fractions were determined by succinate dehydrogenase assay (Fig 1). Then these fractions were run on polyacrylamide gel, transferred on the membrane and detected by polyclonal antibodies of FVIII or monoclonal antibody of Hsp70 as a marker for mitochondria. The results showed in Fig 2 that the factions of FVIII are proportional to the fraction of the mitochondria. The existence for FVIII in mitochondria of mouse liver was determined as well as the co-localization of FVIII and Hsp70. The human data is still in processing.

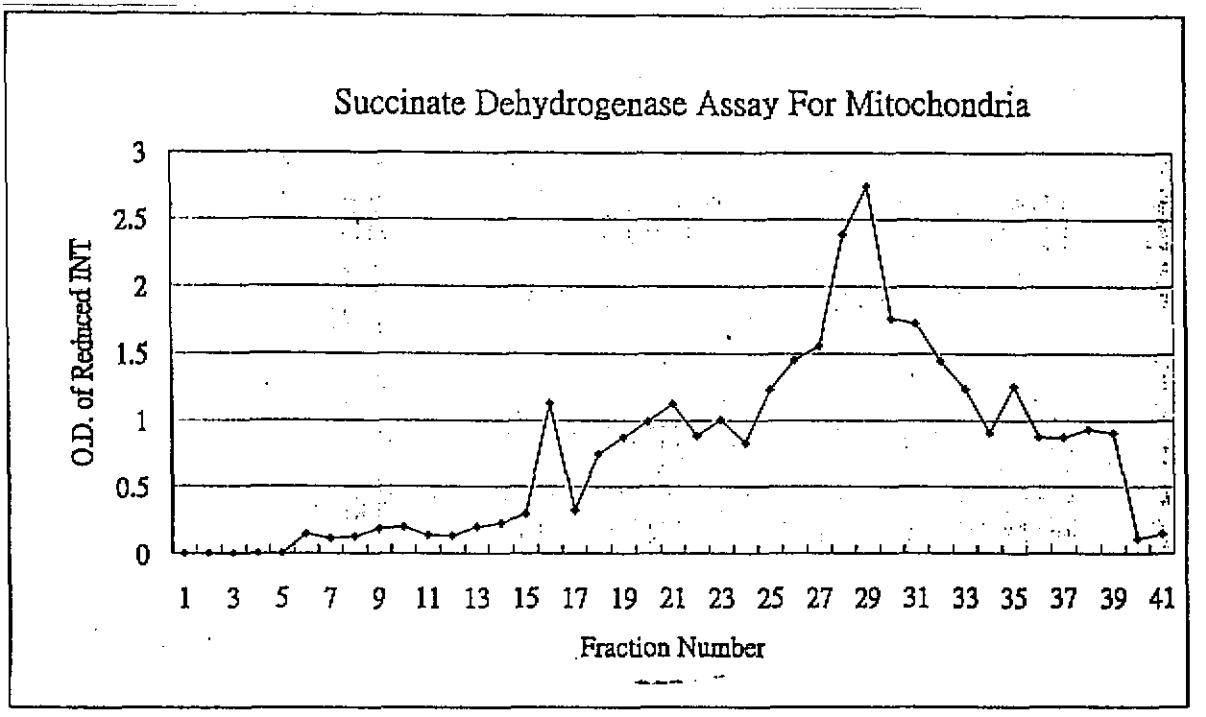

Figure 1 Succinate Dehydrogenase Assay For Mitochondria

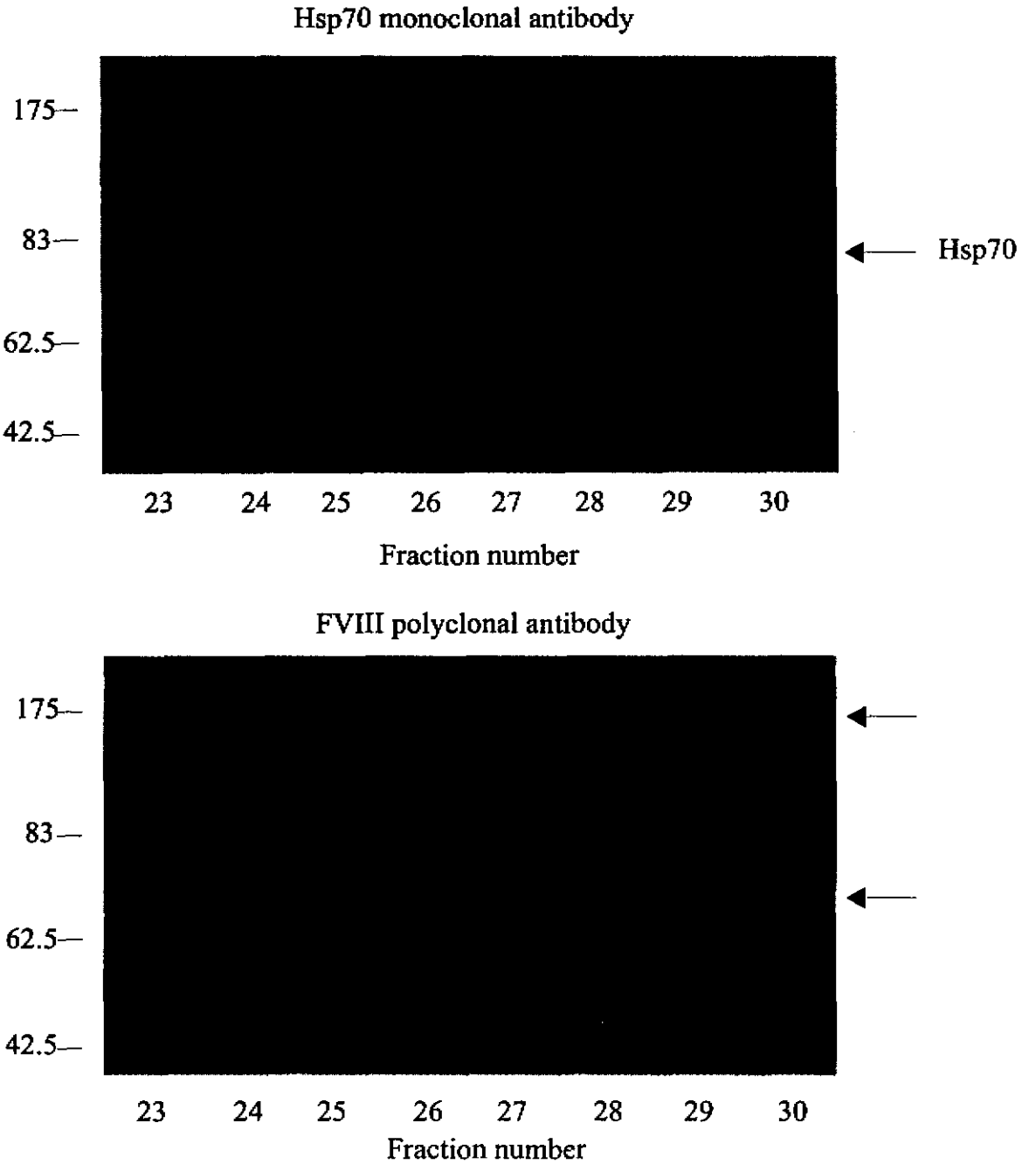

Figure 2 Western blot of sucrose gradient fractions (Hsp70 and FVIII)

patient	F.VIII:C (%)	Mutation Nucleotide	Amino Acid (codon)	Exon / Intron	Severity	Inhibitor
TWN-44	18	GCC → GTC	Ala → Val (1834)	Exon 16	Mild	
TWN-46	17	CGA → CAA	Arg → Gln (1941)	Exon 18	Mild	
TWN-100	7.2	CTT → TTT	Leu → Phe (1789)	Exon 16	Mild	
TWN-7	1	GCT → CCT	Ala → Pro (2192)	Exon 24	Moderate	
TWN-86	4.6	ATG → ATA	Met → Ilu (1823)	Exon 16	Moderate	
TWN-4	<1	ATT → AGT	Ile → Ser (386)	Exon 8	Severe	
TWN-18	<1	GGT → CGT	Gly → Arg (111)	Exon 3	Severe	
TWN-43	<1	CGC → TGC	Arg → Cys (1689)	Exon 14	Severe	
TWN-64	<1	TGG → TGC	Trp → Cys (585)	Exon 12	Severe	
TWN-66	<1	GAG → AAG	Glu → Lys (1885)	Exon 17	Severe	
TWN-68	<1	TAC → TCC	Tyr → Ser (586)	Exon 12	Severe	Yes
TWN-72	<1	GGG → GAG	Gly → Glu (1760)	Exon 15	Severe	
TWN-106	<1	CCA → TCA	Pro → Ser (146)	Exon 4	Severe	
TWN-13	<1	AAA → TAA	Lys → stop (1827)	Exon 16	Severe	Yes
TWN-15(33)	<1	TAT → TAA	Tyr → stop (323)	Exon 8	Severe	
TWN-17	<1	AAA → TAA	Lys → stop (1827)	Exon 16	Severe	Yes
TWN-45	<1	CGA → TGA	Arg → stop (1966)	Exon 18	Severe	
TWN-65	<1	TGG → TAG	Trp → stop (1942)	Exon 18	Severe	
TWN-82(77)	<1	CGA → TGA	Arg → stop (2116)	Exon 22	Severe	
TWN-91	<1	CGA → TGA	Arg → stop (1966)	Exon 18	Severe	
TWN-93	<1	CAG → TAG	Gln → stop (1796)	Exon 16	Severe	Yes

Table 1: Summary of the molecular defects of the hemophilia A patients

Cell type	**Amount of vWF release (% of normal in plasma)**
HUVECs: Wash with PBS, refresh with HUVEC growth medium in 10% fetal bovine serum for overnight, then refresh with serum free medium overnight.	10
HUVECs: Wash with PBS, refresh with HUVEC growth medium in 2% fetal bovine serum for overnight, then refresh with serum free medium overnight	3
293 cells: Wash with PBS, refresh with DMEM growth medium in 10% fetal bovine serum for overnight, then refresh with serum free medium overnight.	0
293 cells: Wash with PBS, refresh with DMEM growth medium in 2% fetal bovine serum for overnight, then refresh with serum free medium overnight	0

Table2: HUVECs release vWF in the conditioned medium

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