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Properties of a recombinant bovine tissue factor expressed by *Silkworm* pupae and its performance as an Owren-type prothrombin time reagent for warfarin monitoring

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ABSTRACT

Tissue factor (TF), or thromboplastin, is a glycoprotein that triggers the extrinsic coagulation pathway. In blood coagulation testing, TF has been used as a natural source for determining Quick prothrombin time (PT) or the Owren PT (OBT). Currently, natural sources are being replaced with recombinant proteins because of their uniform characteristics and the possibility of stable mass production of PT reagents. Because bovine spongiform encephalopathy (BSE)-infected cows are widespread in Japan, we prepared a recombinant bovine TF (rbTF) with a baculovirus expression system using silkworms. To overcome the limitations of natural TF, especially in bovine brain, we expressed a full-length rbTF protein in *Silkworm* pupae with a baculovirus expression system. Baculovirus inactivation and the presence of DNA fragments in the rbTF fraction were confirmed using Reed-Muench and polymerase chain reaction methods after inactivation with a detergent. The rbTF fraction prepared by an immobilized anti-*Silkworm* pupae fluid protein Sepharose 4B column was identified as a visible band on western blots with a polyclonal antibody against human TF with cross-reactivity with TFs. The inhibition of the polyclonal antibody against human TF by the clotting assay for PT was identified, and amidolytic biological activity through activated factor VII on S-2288 substrate was observed. In conclusion, the rbTF expressed by the baculovirus system using *Silkworm* pupae was uniformly specific for bovine TF. The OBT reagent incorporated by this rbTF was similar to those of commercial reagents. It also showed a suitable International Sensitivity Index and reproducibility precision, thereby allowing for diagnostic use.

Introduction

Currently, the 2 following coagulation tests for screening extrinsic pathways and monitoring warfarin are widely used in clinical laboratories: (i) the Quick method [1], which was the first available test, and (ii) the Owren method [2], which was developed along with the discovery of coagulation factor V in 1948 [3]. The Owren method is utilized mainly in Japan, Scandinavia, Benelux, and some European countries, and its clinical benefits have been described in reports from Scandinavian countries [4–7]. Thromboplastin reagents were prepared from a tissue factor (TF) that was derived from rabbit brain or extracted human placenta. Technical trends for Quick prothrombin time (PT) determinations have also moved to the use of recombinant human TF [8] or recombinant rabbit TF [9,10]. From the perspective of monitoring anticoagulant therapy in order to prevent thrombosis events and decrease bleeding risks, these conventional methods of Quick or Owren PT testing are popular. Warfarin is the most widely used drug for oral anticoagulant therapy (OAT). The original Quick PT technique measures changes in the vitamin K-dependent coagulation factor II (prothrombin), factor VII, and factor X, which are the targets of warfarin. However, it is also dependent on the levels of factor V and fibrinogen (factor I) [1]. In contrast, the Owren PT (or thromboplastin combined) technique measures the total levels of factor II, factor VII, and factor X. Absorbed bovine plasma that is treated with Barium ions is used as a source for adding fibrinogen (factor I) and factor V to the Owren PT reagent (OBT) [2]. Laboratory variations have been a constant issue, but an important harmonizing step was established in 1983 when the World Health Organization (WHO) recommended the use of the International Sensitivity Index (ISI) and the International Normalized Ratio (INR) as the preferred procedure for standardization [11,12]. Primarily, the original International Reference Preparation (IRP) for Thromboplastin reagents was the Thromboplastin combined (coded 67/40), which was prepared from a human origin and complementary to fibrinogen and factor V. The Quick PT or OBT testing that is expressed as INR is the standardized measurement procedure that is used to monitor OAT. This depends on the calibration of individual thromboplastin reagents against the WHO's IRP for thromboplastin reagents. However, the narrow therapeutic range in INR involves possible dangers of serious complications and bleeding risks or thrombosis events.

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Nevertheless, the use of INR/ISI for monitoring warfarin is currently mandatory in Japan and in many other countries. Originally, it was thought that the INR system would decrease dependence on the commercial PT reagent's sensitivity and that OAT therapeutic ranges for care would be uniform between laboratories. In practice, the use of the INR system involves differences in citrate concentrations in plasma specimens, thromboplastin origins, detection principles or algorithms of coagulation analyzers, and local ISI calibrations to harmonized INR results. With such a background, OBT measurements have improved assay precision, both between laboratory coefficients of variability (CV) [4–6] and the therapeutic targeted INR range (INR 2-3) that was measured by OBT testing, and may represent a safety line between thrombosis events and bleeding risk. During the harmonization of Quick and OBT measurements, rabbit and bovine brains became the predominant sources of commercial reagents. However, bovine brain sources were discontinued when bovine spongiform encephalopathy (BSE) became an issue. Bovine brain, which is a BSE-affected organ, has been banned by notice from the Japanese government since 2001. For this reason, bovine brain bulk, imported from New Zealand or other non-infected countries (without BSE risks), and rabbit brain bulk, which is used as an alternative source, are available. Bovine plasma for diagnostic use is available after a confirmation test of BSE safety in Japan. However, the BSE risk is not totally removed as bovine plasma is still used. Currently, in Scandinavian countries, Benelux, parts of Europe (Austria, Russia, etc.), and Japan, commercially available OBT reagents, such as STA-SPA (Roche, France), HepatoQuick (Stago), Pro-complex (Instrumentation Laboratory, Italy), Owren-PT (MediRox, Finland), and Thrombotest (Axis-Shield plc, Scotland), are still used as bovine citrated plasma bulk. In this study, we set out to find an alternative for natural bovine brain TF that involves a recombinant bovine TF (rbTF) with a stable supply and that reduces BSE risk. We focused on a unique protein expression system using the baculovirus expression systems hosted by *Silkworm* pupae, by which a high yield, biological activity, and high throughput are expected, in order to produce an rbTF that has bioactivity and potential for our target [13,14]. Subsequently, we investigated whether the rbTF could be applied as a bulk for OBT reagents.

Materials and Methods

Reagents

Chemical Materials

All chemical reagents were used the highest grade available commercially. CNBr-Sepharose CL-4B and Protein A column was obtained from GE Healthcare, Tokyo, Japan. 1,2-Dioleolyl-sn-glycero-3-phosphoethanolamine (PE : MW 765), 1,2-dioleolyl-sn-glycero-3-phosphocholine (PC : MW 711), and 1,2-dioleolyl-sn-glycero-3-phospho-L-serine sodium (PS : MW 811) were obtained from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Activated factor VII (FVIIa) was used a “NovoSeven” drug (Novo Nordisk Pharma Ltd, Japan). Chromogenic substrate S-2288 (Chromogenix Instrumentation Laboratory S.p.A, Milano, Italy), which has a hydrolytic cleavage with the complex of TF and activated factor VII, was used. For electrophoresis analysis, 5%–20% polyacrylamide gel, Mini-PROTEAN system, and colorimetric HRP substrate reagent kit were used from Bio-Rad Laboratories, Inc (Japan). For the western blot analysis, PVDF membrane and iBLOT transfer device were used from Life Technologies Corporation (Japan).

Biological Materials and Diagnostic Reagents

Silkworm pupae were obtained from Katakura Industries Co, Ltd (Tokyo, Japan). The recombinant expression system was used the ProCube service (Sysmex Corporation, Kobe, Japan). Goat anti-human TF polyclonal antibody (product 4501) (American Diagnostica Inc, Stamford, CT, USA), which has a cross reactivity to human, rabbit, and bovine species, was used. Secondary antibody; anti-Goat IgG (H + L-chain)-HRP Rabbit IgG/Fab' (Medical & Biological Laboratories Co, Ltd, Nagoya, Japan) was used. A commercial recombinant human TF apoprotein (Eukaryote; Altor BioScience Corporation, USA) and recombinant rabbit TF apoprotein (*E coli*; Pel-Freez Biologicals, Rogers, AR, USA) were used as a reference. Fresh Bovine brain was imported from New Zealand by Japan Ram Co, Ltd (Hiroshima, Japan) in cold storage. The Commercial OBT reagent, Thrombotest owren, was purchased from Eisai-Eidia Co, Ltd (Tokyo, Japan). In order to prepare the OBT reagent using the recombinant bovine tissue factor (rbTF), frozen bovine citrate plasma was purchased from DADO Corporation (Hokkaido-Obihiro, Japan). The bovine blood treated with less than two hours after slaughter was mixed with 3.2% (w/v) sodium citrate at a ratio of 1:9 below 15 degree according to the regulation of Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF).

Certified Plasma Materials and Local Calibration

A standard human plasma, Coagtrol N (Sysmex Corporation), was used for normal level plasma. AK-CALIBRANT plasma materials composed from 1 normal plasma (AK-A), 3 warfarin plasmas (AK-B, AK-C, AK-D) (Technoclone, Austria) [15] was used for the precision study. The ISI value was determined by AK-CALIBRANT plasma material, which was assigned mean INR in package insert. It was calculated by logarithmic linear regression equation by the plot of seconds and assigned INR in AK-CALIBRANT plasmas, and was basically used according to the Local Calibration (L-C) method [16], not used by Local SI according to WHO's International reference preparation (IRP).

Preparation of IgG Fraction from Anti-Silkworm Pupae Proteins, Rabbit Serum, and Immunoabsorbed Columns

Rabbit anti-*Silkworm* pupae fluid serum was prepared by immunizing rabbits with multiple subcutaneous injections of 0.1 mg of *Silkworm* pupae whole protein solution in Freund's complete adjuvant at 2-week intervals. The rabbits were bled 10 weeks later. The rabbit serum obtained from blood of rabbits was treated twice with the Protein A column (GE Healthcare), and a IgG fraction adsorbed to the Protein A column was purified according to the instruction manual. The rabbits' serum was completely free from traces of contaminant antibody. The IgG fraction was purified from the above rabbit anti-serum with Protein A column chromatography. The immunoabsorbed column with IgG from anti-serum against *Silkworm* pupae proteins was prepared by the coupling process to CNBr-activated Sepharose CL-4B (GE Healthcare) according to the manufacturer's instructions.

Cloning of cDNA Library

cDNA of bTF was amplified from a bovine brain cDNA library (Agilent Technologies, Inc, Santa Clara, CA, USA). The primer pair was 5′-GGATCCATGGCGACCCCCAACGGGCCCCG-3′ (forward primer with *Bam*HI site) and 5′-CTCGAGTTATGCAGCGTTGAGCGGCGTG-3′ (reverse primer with *Xho*I site). Amplified products were cloned into a TA cloning vector with a TOPO TA Cloning Kit (Life Technologies, Grand Island, NY, USA). The cDNA insert was sequenced on a 4200L DNA sequencer (LI-COR).

Fractionation of Recombinant bTF (rbTF)

In order to obtain a fraction of rbTF protein, it was expressed using a baculovirus-*Silkworm* pupae expression system (ProCube, Sysmex Corporation) as follows. Primers UXhomerC (5′-CCGCTCGAGATGGG ACTGATGACACGCATT-3′) and LXbamerChistag (5′-GCTCTAGATTAG TGATGGTGATGGTGATGCAAGCGCTTGGCGGGGAG-3′) or UXhomerE (5′-CCGCTCGAGATGAACGCCCCTGACAAAC-3′) and LXbamerEhistag