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Characterization of recombinant murine factor VIIa and recombinant murine tissue factor: a human—murine species compatibility study

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Abstract Tissue factor (TF) is believed to play an important role in coagulation, inflammation, angiogenesis and wound healing as well as in tumor growth and metastasis. To facilitate in vivo studies in experimental murine models, we have produced recombinant murine factor VII (FVII) and the ectodomain of murine TF, TF(1—223). Murine FVII was activated to FVIIa with human factor Xa and upon reaction with FFR-chloromethyl ketone converted into an active site-blocked TF antagonist, FFR-FVIIa. The activity of murine FVIIa was characterized in factor X activation assays as well as in clot assays with murine and human thromboplastin in murine and human plasma. In these assays murine FVIIa exhibited a specific activity equivalent to or higher than human FVIIa. Further analysis showed that murine FVIIa binds with high affinity to both murine and human TF, whereas the association of human FVIIa to murine TF is about three orders of magnitude weaker than the association to human TF. This difference was further emphasized by the effect of murine-and human FFR-FVIIa on bleeding in an in vivo mouse model. Intra-peritoneal administration of 1 mg/kg murine FFR-FVIIa significantly prolonged the tail-bleeding time, whereas no effect on bleeding was observed with a 25-times higher dose of the human FFR-FVIIa. Together, these data confirms the notion of poor species compatibility between human FVII and murine TF and emphasizes the requirement for autologous FVIIa in studies on the role of the TF in experimental in vivo pharmacology.

Abbreviations: TF, tissue factor; FVII, factor VII; FVIIa, factor VIIa; FFR-FVIIa, FVIIa blocked in the active site with phenylalanyl-phenylalanyl-arginyl-chloromethyl ketone; FX, factor X; FIX, factor IX; PCR, polymerase chain reaction; EDTA, ethylenediaminetetra-acetic acid; BSA, bovine serum albumin; IgG, immunoglobulin G; CHO cells, Chinese hamster ovary cells; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PT, prothrombin time; APTT, activated partial thromboplastin time.

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Introduction

Tissue factor (TF) functions as the cellular receptor for plasma factor VIIa (FVIIa), and the formation of TF-FVIIa complexes on cell surface membranes triggers the coagulation cascade. Exposure of TF on cell surfaces results in binding of FVIIa and subsequent activation of factor X (FX) and factor IX (FIX). The activated factors, FXa and FIXa, then activate downstream coagulation factors resulting ultimately in thrombin-mediated fibrin deposition (see Refs. [1—3] for review). TF is present on fibroblasts and smooth muscle cells surrounding the vessels but normally not on cells in contact with the blood. However, it can be exposed to FVII in the circulation upon vessel injury/leakage or due to up-regulation of TF on monocytes or endothelial cells as a result of an inflammatory reaction. The organization of the TF promoter [4] implies that TF can be up-regulated in response to various injuries, and thus induction of TF expression may be an important part of a number of normal and diseased states including tissue remodeling, wound healing, inflammation, thrombosis, disseminated intravascular coagulation and cancer.

The current focus on TF and its expression in experimental in vivo models makes the understanding of its interaction with FVII/FVIIa all the more important. Murine models are attractive in such studies for obvious reasons. The size of the animal, the ease of breeding, the availability of the entire genome and the potential for generating gene knock-out animals provide some of the attractive features of murine models. However, functional implications of species differences have been an obstacle to in vivo TF biology studies in mice. That human—mouse species differences constitute a particular problem was shown early by two groups who noted that TF-containing murine cell homogenates [5] or murine thromboplastin preparations [6] were poor initiators of coagulation in human plasma. Impaired binding of human FVIIa to the region in mouse TF coded by exon 3 was subsequently proposed as the reason for this species incompatibility [7]. Rabbit FVII [8] and rat FVII [9] have previously been expressed and purified for use in experimental in vivo pharmacological studies. To facilitate in vivo studies on the role of TF in normal and diseased states we report the expression, purification and characterization of recombinant murine FVII and the ectodomain of murine TF.

Materials and methods

Reagents

D-Phenylalanyl-L-phenylalanyl-L-arginyl-chloromethyl ketone (FFR-cmk) was from Bachem (Bubendorf, Switzerland), S-2288 and S-2765 from Chromogenix (Mölndal, Sweden) and Chromozyme X from Boehringer Mannheim (Ingelheim, Germany). Human FVIIa and FFR-FVIIa was from Novo Nordisk (Bagsværd, Denmark), human FXa and FX were from Enzyme Research Laboratories (South Bend, IN, USA), mouse B16-F10 melanoma cells were purchased from Riken Cell Bank (Tsukuba, Japan). Human Recombinant TF(1—219) was expressed in *E. coli* and purified as described [10].

Cloning and expression of murine TF(1—223)

The gene encoding murine TF was described by Ranganathan et al. [11]. Using the primers mTF forward (5′ -GTGATC*CATATG*GCAGGCATTCCAGAGAAAGCG-3′) and mTF reverse (5′ -AAT*GGATCC*TCATTCTCCCAGGAAACTCTTCCATTG-3′) and the full-length cDNA for mTF a product corresponding to mTF(29—251) (subsequently designated mTF_{1-223}) was obtained using the expand high fidelity PCR kit from Roche according to the manufactures directions. The two primers contain an *Nde*I and a *Bam*HI site (underlined), respectively, and these restriction sites were used to introduce the gene into the corresponding sites of the pET-3a expression vector. The sequence of the entire insert was verified by DNA sequencing using the dye-deoxy method and equipment from Applied Biosystems. The generated plasmid encoding mTF_{1-223} was then introduced in the *E. coli* BL21 (DE3) strain for expression. Expression was carried out as follows: 10 ml of sterile LB medium containing 100 μg/ml carbenicillin was inoculated with freshly transformed BL21 (DE3) cells harboring the mTF_{1-223} pET-3a plasmid and incubated over night at 37 °C, 250 rpm. The following day four 1-l bottles containing 400 ml of sterile LB medium containing 100 μg/ml carbenicillin was inoculated with 1 ml of the overnight culture and grown at 37 °C, 250 rpm to OD_{600}=0.6—0.8 at which point expression was induced by addition of IPTG to a final concentration of 1 mM. Following induction of expression, the temperature and rate of shaking was reduced to 30 °C and 200 rpm and incubation continued overnight.

Following expression, cells were harvested by centrifugation and resuspended in 100 ml 50 mM Tris, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, pH 8.0 and lysed by sonication.