Generation of a polyclonal rabbit anti-mouse tissue factor antibody by nucleic acid immunisation

Andrew D. Mumford[1], Daxin Chen[2], Anthony Dorling[2], Geoffrey Kemball-Cook[1], John H. McVey[1]

[1]Haemostasis and Thrombosis, MRC Clinical Sciences Centre and [2]Department of Immunology, The Faculty of Medicine, Imperial College, Hammersmith Hospital Campus, London, United Kingdom

Summary

Tissue factor (TF) the cellular receptor and cofactor for factor VII, initiates coagulation and has also been implicated in several coagulation-independent functions, including inflammation, angiogenesis and tumour metastasis. Investigations of TF expression in mouse models of these processes has been limited by the availability of antibodies that specifically recognise mouse TF. We have generated a rabbit polyclonal antibody to mTF by DNA immunisation. This has yielded an antiserum that recognises native mTF in immunohistochemical and flow cytometric analyses. Furthermore, the antiserum is inhibitory in coagulation assays. This antiserum will be a valuable investigative tool in the analysis of mTF expression.

Keywords

Tissue factor, antibody, immunisation

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Introduction

Techniques such as flow cytometry and immunohistochemistry are powerful investigative tools with which to demonstrate the expression of cellular proteins. However, since these techniques employ immunological methods to detect protein expression, their efficacy depends on the availability of an antibody with sufficient sensitivity and specificity for the protein of interest. Investigational antibodies are usually obtained from the sera of host animals immunised with peptide, purified native protein or recombinant protein antigen. However, the preparation and purification of peptides, native and recombinant proteins for immunisation is time-consuming, costly and their immunogenicity is unpredictable.

These difficulties are illustrated by recent attempts in our laboratory to detect mouse tissue factor (mTF). We initially studied mTF using a polyclonal rabbit antibody we had previously raised against recombinant human $TF_{1–219}$ ($hTF_{1–219}$) expressed in *E.coli*, however this antibody showed poor cross-reactivity with mTF (Fig. 1A). We then attempted to express recombinant mouse $TF_{1–225}$ using the same system as we had used for recombinant $hTF_{1–219}$ (1), however no mTF was produced. We then immunised rabbits with synthetic peptides derived from the mTF extracellular domains (including one previously reported to generate an effective anti-mTF antibody (2)). All the peptides induced an immune response and the antisera specifically recognised the linear peptide sequence, however they reacted poorly with mouse TF expressed by murine fibroblasts in Western blot, immunohistochemical and flow cytometric analyses (data not shown). We have now circumvented these problems by using a simple nucleic acid immunisation method to generate a rabbit polyclonal anti-mTF antibody that is effective in detecting both recombinant and native mTF in a variety of experimental settings.

Materials and methods

Generation of expression constructs

An amplicon containing either the full-length mTF coding sequence (1–294) or a truncated sequence encoding the extracellular domains

Correspondence to:
Dr John McVey
Haemostasis and Thrombosis
MRC Clinical Sciences Centre
The Faculty of Medicine, Imperial College
Hammersmith Hospital Campus
Du Cane Road
London, W12 0NN
United Kingdom
Tel: 020 8383 8253, Fax: 0870 131 3540
E-mail: john.mcvey@csc.mrc.ac.uk

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of mTF (1–225) were generated by PCR using the sense oligonucleotide 5'-AGATCTGGACATGGCGATCCTC-3' and the antisense oligonucleotides 5'-GGATCCCCCTATGCCAAGCGCG-3' or 5'-AGATCT**CTA**TTCTCCCAGGAAACTC-3' respectively. The termination codon introduced into the $mTF_{1–225}$ is shown in bold. The oligonucleotide primers incorporated sites for *Bgl* II and *Bam*H I (underlined) to facilitate cloning into the *Bam*H I site of the mammalian expression vector pcDNA3.1 (Invitrogen, Paisley UK). The sequence of the mTF amplicons was verified by DNA sequence analysis. The plasmid expression vectors were transformed into *E.coli* competent cells and these were expanded in a large-scale culture from which endotoxin-free plasmid DNA was purified (Endofree Plasmid Mega kit; Qiagen, Crawley, UK). The amount of endotoxin present was quantified by a limulus amoebocyte-clotting assay (Pyrogent Plus; BioWhittaker UK, Wokingham, UK).

Immunisation protocol

Two hundred µg of purified plasmid in 200µl of phosphate buffered saline were injected without adjuvant into each quadriceps muscle of the hind legs of two New Zealand white rabbits (Harlan Sera-Lab, Loughborough, UK). Booster injections were similarly given at 28 and 56 days and a terminal bleed was carried out at 70 days. Pre-immune serum samples were obtained prior to the primary immunisation.

Slot blot analysis

Aliquots of *E.coli* recombinant human $TF_{1–219}$ (produced in-house) and mouse $TF_{1–223}$ (a kind gift of Dr J. Morrisey, University of Illinois) were applied directly to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK) using a slot blot device. Non-specific antibody binding sites were blocked for 30 minutes with blocking buffer (Superblock; Perbio Science, Tattenhall UK). The antisera were applied to the membranes (1:1000 dilution) for 60 minutes. After washing, a goat anti-rabbit IgG-HRP conjugate (BioRad, Hemel Hempstead, UK) was applied (1:5000 dilution) for 30 minutes. The membranes were analysed using the washing and antibody buffers from an UnBlot™ In-Gel Chemiluminescent detection kit and visualised with SuperSignal™ West Pico Chemiluminescent substrate (Perbio Science).

Immunohistochemistry

Tissue was embedded in optimal cutting temperature (OCT; VWR international, Poole, UK) by freezing with dry ice, sectioned and fixed in methanol at –20°C. Frozen sections were immersed in 1% bovine serum albumin-phosphate buffered saline and 10% goat serum (Sigma, Poole, UK) for 30 minutes and then incubated overnight at 4°C with rabbit anti-mTF serum or rabbit polyclonal anti-mouse α-actin (Sigma). Second layer staining was with goat anti-rabbit IgG-FITC (Sigma). Sections were examined on an immunofluorescence microscope (Axiovert S100 TV; Zeiss, Welwyn Garden City, UK) with Plan-NEOFLUAR objectives using a KTL/CCD-1300/Y/HS camera from Princeton Instruments (Trenton, NJ). Images were analysed using the MetaMorph imaging system (Universal Imaging, Downington, US).

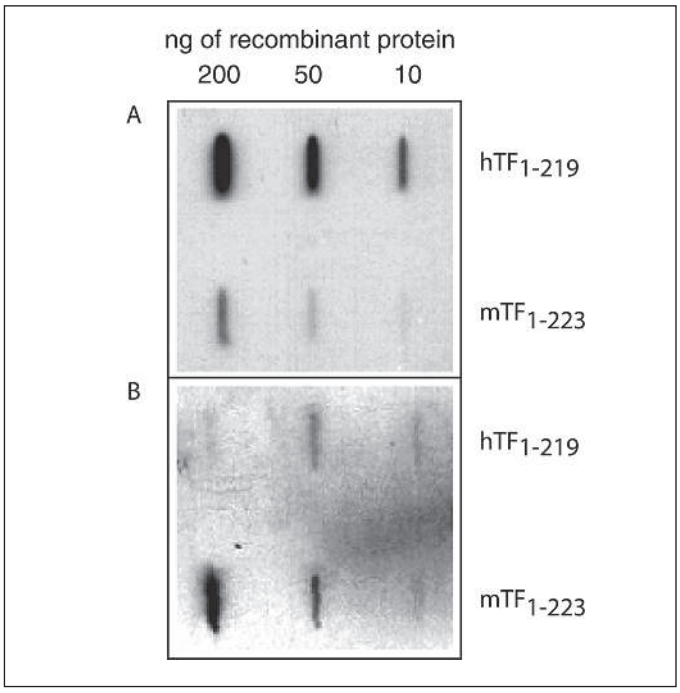

Figure 1: Slot blot analysis of $hTF_{1–219}$ and $mTF_{1–223}$ with an anti-$hTF_{1–219}$ antibody and the anti-$mTF_{1–225}$ serum. Aliquots of 200, 50 and 10 ng of $hTF_{1–219}$ and recombinant mouse $mTF_{1–223}$ were applied to a nitrocellulose membrane using a slot-blot device. The membranes were probed with either (A) rabbit anti-$hTF_{1–219}$ antibody or (B) rabbit anti–$mTF_{1–225}$ serum generated by DNA immunisation. The membranes were then treated with a secondary goat anti-rabbit IgG antibody conjugated to horseradish peroxidase and were visualised using a chemiluminescent detection system.

Purification of mouse microvascular endothelial cells

Endothelial cells (EC) were purified as previously described (3). Briefly, cells were isolated by positive selection using rat anti-mouse CD31 and CD105 (Pharminogen, Oxford, UK) and goat anti-rat microbeads (Miltenyi Biotec, Auburn, USA). All ECs were passage 1. ECs were activated by thrombin (1 iu/ml; Enzyme Research Laboratories, Swansea, UK) for 12 hours at 37°C prior to analysis.

Analysis of monocytes from lipopolysaccharide (LPS) treated mice

All mice weighed 25+/-1g. Experiments were performed under terminal anaesthesia (Sagatal; 60ng/g, Rhone Merieux, Harlow, UK). Mice were given a single injection of LPS (*E.coli* serotype 0127:B8; Sigma) 2µg/g or saline intraperitoneally. Nitro-L-arginine methyl ester (Alexis, Nottingham UK) 50µg/g was administered intraperitoneally 30 minutes before LPS and again 0, 2 and 4 hours after LPS. Mouse blood, collected by cardiac puncture 4.5 hours after LPS or saline administration, was diluted 1:20 in 155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA to lyse erythrocytes. After 20 minutes at room temperature, cells were washed and resuspended in 3% foetal calf serum in Dulbecco modified Eagle medium (Sigma). The monocyte phenotype was confirmed by flow cytometric analysis with an anti-mouse CD14 antibody (Pharmingen).

Flow cytometry analysis

Cells were incubated with primary antibodies for 30 minutes. Stained cells were analysed on a FACSCAN flow cytometer (Becton Dickinson, CA).

Clotting assays

Cell surface procoagulant activity was measured in transfected CHO populations after detaching cells with phosphate buffered saline containing 5mM EDTA. 50µl aliquots of cell suspensions (1 x 10^6 cells/ml) in TBSA (50mM Tris pH 7.4, 150mM NaCl, 1mg/ml clinical grade human albumin; Bio Products Laboratory, Elstree, UK) were added to 50µl of citrated mouse plasma (Sigma) and 50µl of rabbit brain phospholipids (Bell and Alton platelet substitute; Diagnostic Reagents, Thame, UK) and clotting times were measured manually after the addition of $CaCl_2$ to 6.3mM. Clotting times were determined in duplicate and results were quantified from a standard curve prepared from a series of dilutions of relipidated recombinant human $TF_{1–243}$ (a kind gift of Dr DL Eaton, Genentech Inc, San Francisco USA) and expressed as a procoagulant activity equivalent to ng human $TF_{1–243}$ per 10^7 cells.

For pre-incubation assays using mouse plasma, 50µl diluted serum in TBSA were added to 200µl mouse TF reagent (saline suspension of mouse brain acetone powder, Sigma B-9136) and incubated at room temperature for 15 minutes. 80µl were added to 80µl citrated mouse plasma (Harlan Sera-Lab) and clotting times were measured manually at 37°C after the addition of 80µl 25mM $CaCl_2$. Results were quantified using a standard curve prepared from mouse TF reagent diluted with TBSA.

For pre-incubation assays using human plasma, 40µl serum diluted in TBSA were added to 300µl mouse TF reagent and incubated at 37°C for 90 minutes. 100µl were added to 100µl citrated human FVII deficient plasma (Diagnostic Reagents) containing recombinant human FVIIa at a final concentration in the assay of 100nM and clotting times were measured manually at 37°C after the addition of 100µl 25mM $CaCl_2$. Results were quantified from a standard curve prepared from mouse TF reagent diluted with TBSA.

Results

Nucleic acid immunisation is a technique in which a plasmid expression vector containing a cDNA encoding the desired peptide antigen is administered to a mammalian host by intra-muscular injection leading to uptake of the cDNA and expression of the peptide by host cells (4). In order to ensure that the mTF peptide was expressed as a structurally intact trans-membrane protein in heterologous mammalian cells, we first transfected Chinese hamster ovary (CHO) cells with the full-length mTF coding sequence in the mammalian expression vector pcDNA3.1. We tested the procoagulant activity of the stably transfected CHO cell lines by measuring the ability of cell suspensions to accelerate fibrin clot formation in mouse plasma. Cell lines transfected with the full-length mTF cDNA showed greater procoagulant activity (equivalent to 175 ng $hTF_{1–243}/10^7$ cells) than cell lines transfected with the expression vector alone (equivalent to 5ng $hTF_{1–243}/10^7$ cells), demonstrating that the mTF expressed by transfected CHO cells was functionally active and therefore structurally intact. mTF expressed using this system was therefore likely to display similar surface antigenic determinants to native mTF. Antibodies generated in the rabbit host to this expressed recombinant mTF are also therefore likely to recognise native mTF on murine cells.

A second expression construct was then assembled comprising a truncated mTF cDNA (encoding amino acid residues 1–225) in pcDNA3.1 (pcDNA3.1-$mTF_{1–225}$). Since the construct did not encode the mTF transmembrane domain or cytoplasmic tail, the construct was predicted to direct expression of only the extracellular domain that would be secreted into the extracellular space.

Endotoxin free (<0.18U/mg DNA) DNA (pcDNA3.1-$mTF_{1–225}$) was prepared and administered to rabbits (n=2) by injection into each quadriceps muscle of the hind legs followed by booster doses 28 and 56 days later. Serum was harvested at 70 days and tested for binding to native recombinant $hTF_{1–219}$ and recombinant $mTF_{1–223}$ on nitrocellulose membranes. The serum contained IgG that bound to both $hTF_{1–219}$ and $mTF_{1–223}$ (Fig. 1B), however the interaction of the antiserum with $mTF_{1–223}$ was demonstrably stronger.

To demonstrate the use of the serum to detect mTF in tissue sections the anti-$mTF_{1–225}$ serum was used to study mTF ex-

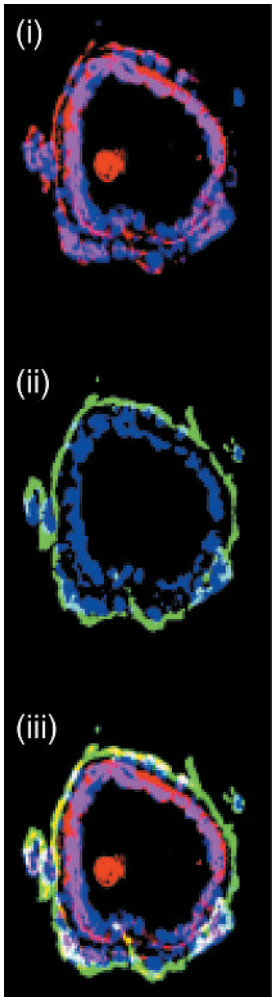

Figure 2: Immunohistochemical analysis using the anti-$mTF_{1–225}$ serum. Frozen sections of mouse carotid arteries fixed in methanol were probed with either a rabbit polyclonal anti-mouse α-actin antibody or anti-$mTF_{1–225}$ serum. The tissue sections were visualised by fluorescence microscopy after treatment with FITC-conjugated goat anti-rabbit IgG secondary antibody. (i) Anti-mouse α-actin is shown in purple (ii) the anti-$mTF_{1–225}$ antiserum in green (iii) merged image of anti-mouse actin and anti-$mTF_{1–225}$ staining; nuclei are stained blue with DAPI.

pression in mouse carotid artery sections using standard immunohistochemistry techniques. TF is constitutively expressed in various tissues, including the adventitial layer but is not expressed by the endothelial (intima) or smooth muscle cell (media) layers of blood vessels (5). Mouse carotid artery sections showed strong selective staining of the adventitia with the anti-$mTF_{1–225}$ rabbit serum (Fig. 2) that was easily distinguishable from the vascular media stained with a monoclonal mouse anti α-actin antibody.

To further demonstrate the applicability and specificity of the serum to detect mTF, microvascular ECs and monocytes were analysed using flow cytometry. Resting ECs do not express TF, however thrombin stimulation *in vitro* has been shown to induce expression of TF (6–8). Similarly, circulating monocytes do not express detectable TF, however following induction of endotoxemia by administration of LPS, monocytes have been shown to stain strongly for TF expression (9, 10). Flow cytometric analysis of unstimulated mouse microvascular endothelial cells stained with anti-$mTF_{1–225}$ serum showed no fluorescence above that of a negative control antibody (polyclonal rabbit anti-human ACTH) however after *in vitro* activation with thrombin, increased fluorescence was observed with the anti-$mTF_{1–225}$ serum but not with the anti-ACTH antibody (Fig. 3A). Flow cytometric analysis of monocytes (CD14 positive) from mice treated with lipopolysaccharide for 4.5 hours demonstrated strong positive staining with the anti-$mTF_{1–225}$ serum in comparison with monocytes isolated from mice treated with saline (Fig. 3B).

The ability of the antiserum to inhibit the functional activity of mTF was then assayed using two different systems. mTF does not promote coagulation of human plasma, however it does promote the coagulation of human plasma to which pre-activated human FVIIa has been added. Increasing amounts of either anti–$mTF_{1–225}$ serum or preimmune serum were preincubated with mouse TF reagent then tested for their ability to promote the coagulation of human FVII deficient plasma containing recombinant human FVIIa (Fig. 4A). The anti-$mTF_{1–225}$ serum inhibited coagulation by up to 98% in a concentration dependent manner. In contrast the pre-immune serum showed slight inhibition only at very high concentrations. The ability of the antiserum to inhibit mouse TF reagent-initiated coagulation of mouse plasma was also tested (Fig. 4B). In this case the anti-$mTF_{1–225}$ serum inhibited coagulation by up to 90% in a dose dependent manner. The pre-immune serum once again did not inhibit coagulation except at very high concentrations.

The ability of the anti-$mTF_{1–225}$ serum to detect a protein of the appropriate molecular weight in Western blot analysis of mouse cells known to express functional TF was also tested. However, the antiserum did not detect a protein following SDS-PAGE under either reducing or non-reducing conditions (data not shown). The immuno-dominant epitope detected by the anti-

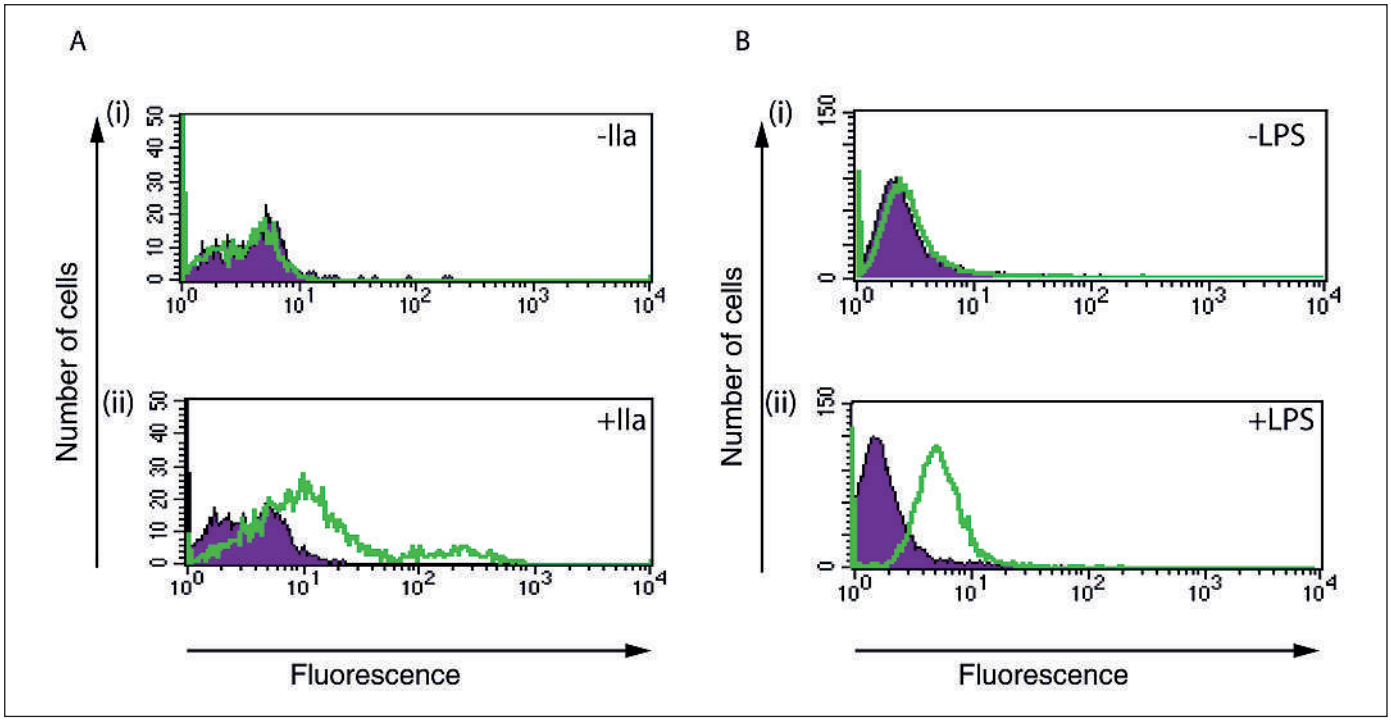

Figure 3: Flow cytometric analysis using the anti-$mTF_{1–225}$ serum. A. Flow cytometric analysis of primary cultures of mouse microvascular endothelial cells cultured for 12 hours in either (i) medium alone or (ii) medium containing 1 iu/ml human thrombin. The cells were incubated with either anti–$mTF_{1–225}$ serum (green trace) or control rabbit polyclonal anti-human ACTH antibody (filled purple trace). The cells were then incubated with a FITC-conjugated goat anti-rabbit IgG secondary antibody and analysed using a FACSCAN flow cytometer. 3000 cells were analysed and the mean fluorescent intensities were (i) 3.78 (anti-ACTH) and 3.19 (anti-$mTF_{1–225}$) and (ii) 3.23 (anti-ACTH) and 11.29 (anti-$mTF_{1–225}$). B. Flow cytometric analysis of monocytes (CD14 positive) isolated from mice treated for 4.5h with either (i) saline or (ii) LPS. The cells were incubated with either anti–$mTF_{1–225}$ serum (green trace) or control rabbit polyclonal anti-human ACTH antibody (filled purple trace). The cells were then incubated with a FITC-conjugated goat anti-rabbit IgG secondary antibody and analysed using a FACSCAN flow cytometer. 10000 cells were analysed and the mean fluorescent intensities were (i) 2.31 (anti-ACTH) and 2.54 (anti-$mTF_{1–225}$) and (ii) 1.86 (anti-ACTH) and 5.50 (anti-$mTF_{1–225}$).

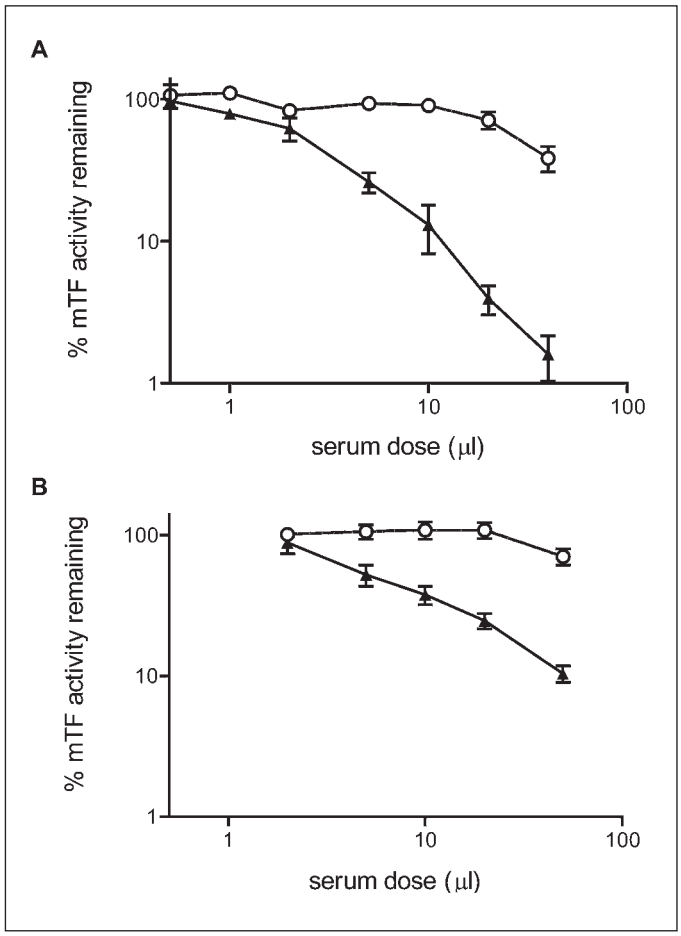

Figure 4: Prolongation of coagulation by anti-mTF$_{1–225}$ serum. A. Effects of anti-mTF$_{1–225}$ serum on mTF-dependent coagulation in human plasma. Increasing amounts of anti-mTF$_{1–225}$ serum were pre-incubated with mouse TF reagent for 90 minutes prior to adding human citrated FVII deficient plasma containing recombinant human FVIIa. Clotting was initiated by addition of $CaCl_2$. The results are the mean of 3 independent experiments (+/-SD). B. Effects of anti-mTF$_{1–225}$ serum on mTF-dependent coagulation in mouse plasma. Increasing amounts of serum were pre-incubated with mouse TF reagent for 15 minutes prior to adding mouse citrated plasma. Clotting was initiated by addition of $CaCl_2$. The results are the mean of 2 independent experiments (+/-SD). Pre-immune serum, open circles; anti-mTF$_{1–225}$ serum, filled triangles.

mTF$_{1–225}$ serum is most likely a conformational epitope sensitive to denaturation following treatment with SDS.

Discussion

Since antigen expressed following nucleic acid immunisation is capable of eliciting an antigen-specific cytotoxic T-cell response in the host, nucleic acid immunisation has become a popular strategy in the development of vaccines against viral and protozoan pathogens (11). However, expression of a cDNA by host mammalian cells also elicits a potent humoral response through uptake of the expressed material by antigen presenting cells and presentation of processed antigen in association with MHC class II molecules to T helper cells. We have exploited these properties of nucleic acid immunisation to generate a polyclonal serum in a rabbit host against the mTF extracellular domain. This approach yielded an antibody that showed specificity for mTF as demonstrated by slot blot analysis of native recombinant human and mouse TF (Fig. 1). The anti-mTF$_{1–225}$ serum could also be used to detect immunoreactivity both by flow cytometric and immunohistochemical analyses that demonstrated expression patterns that concord with previously descriptions of TF expression. Namely, constitutive expression of TF by the adventitial layer of blood vessels but not by the medial or intimal layers (Fig 2) (4); no detectable expression of TF by unstimulated endothelial cells and monocytes but detectable expression following stimulation with thrombin and LPS respectively (Fig. 3) (5–9). Furthermore, the anti-mTF$_{1–225}$ serum was inhibitory in clotting assays further demonstrating the specificity of the antibody (Fig. 4).

Nucleic acid immunisation offers the significant advantages over conventional immunisation strategies using peptides or recombinant protein because synthesis and purification of plasmid DNA expression vectors is simple and rapid. Furthermore, since the plasmid backbone DNA of expression constructs is of prokaryotic origin, the immunised material also contains immunostimulatory CpG motifs capable of enhancing the host humoral response (12). Nucleic acid immunisation therefore represents a powerful technique with potential applications to a range of cell surface proteins. This strategy may be particularly applicable to study peptide antigens that show poor immunogenicity in conventional immunisation techniques.

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