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Short communication

Generation and characterization of a monoclonal antibody against canine tissue factor

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ABSTRACT

Tissue factor (TF, coagulation factor III) has recently identified roles in innate immunity and cancer. We generated a murine mAb against canine TF (cTF) cloned from Madin-Darby canine kidney cells and expressed in Chinese Hamster Ovarian (CHO) cells, with an equine IL-4 tag. One clone was selected for purification based on initial screening of CHO cell supernatants. The mAb was further characterized with flow cytometry, immunofluorescent microscopy, immunoblotting and immunohistochemical staining of normal and neoplastic canine tissue. The mAb labeled high, but not low, TF-expressing canine breast cancer (CMT25) and osteosarcoma (HMPOS) cells with flow cytometry and immunofluorescent microscopy. Immunoblotting revealed a 42 kDa protein with homogenized canine brain and CMT25, but not HMPOS, lysates. The mAb labeled renal tubules and glomeruli, intestinal and dermal epithelium, and arteriolar adventitial cells in frozen tissues. Using immunofluorescent microscopy, increased numbers of labeled PBMCs were observed after LPS stimulation. Our results indicate that the anti-cTF mAb detects a protein with the expected tissue distribution and molecular weight of TF in normal, LPS-stimulated and neoplastic canine cells. This mAb may prove useful for exploring the role of TF in neoplastic and infectious disorders in dogs.

1. Introduction

Tissue factor (TF, also known as coagulation factor III or tissue thromboplastin) is a protein best known for its role in hemostasis, where it is the main trigger of thrombin generation (Mackman, 2009). Tissue factor is normally expressed on perivascular fibroblasts in the adventitia of arteries and activates coagulation on vessel injury. It is also richly expressed in brain parenchyma and various epithelia in human and murine tissues (Drake et al., 1989; Flossel et al., 1994; Luther et al., 1996). In pathological states, inflammatory cytokines and LPS induce TF in monocytes, both on the cell surface and on membrane-derived microvesicles. This induced expression is thought to be the main initiator of disseminated intravascular coagulation in sepsis (Pawlinski and Mackman, 2010). Tumors can also express TF on their membranes or shed microparticles (Andreasen et al., 2014; de la Fuente et al., 2014; Stokol et al., 2011). Tissue factor has been implicated in tumor growth, angiogenesis, metastasis and paraneoplastic thrombosis (Andreasen et al., 2014; Wang et al., 2012). Recent studies have also uncovered roles for TF and hemostasis in immune responses to infectious agents (Antoniak and Mackman, 2014; Armstrong et al., 2013).

Murine mAbs against human TF have been used to detect Ag expression in histological sections of normal and diseased tissues (Drake et al., 1989; Flossel et al., 1994; Luther et al., 1996), capture and quantify monocyte- or tumor-derived microparticles (Lee et al., 2012; Tatsumi et al., 2014), and inhibit TF procoagulant activity (Key and Mackman, 2010). High levels of TF Ag have been correlated with tumor grade, incidence of thrombotic events and prognosis in cancer patients (Geddings and Mackman, 2013).

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Similarly, microparticle TF activity correlates to bacterial LPS load (Hellum et al., 2014) and is associated with coagulation activation in endotoxemic mice (Wang et al., 2009). This indicates that TF antigen measurement could serve as a diagnostic and prognostic biomarker in patients with cancer or bacterial infections. Unfortunately, murine mAbs raised against human TF do not appear to crossreact with the canine protein (Morrissey et al., 1988). Recently, a rabbit anti-TF mAb was used to detect TF in canine gliomas in formalin-fixed tissue (de la Fuente et al., 2014). We and others have also used polyclonal anti-human or anti-canine TF Ab to detect TF in canine cancer cells (Stokol et al., 2011) or tissues (Andreasen et al., 2014), however, polyclonal Ab are not readily adaptable for capture-based ELISAs used for TF measurement in plasma (Key and Mackman, 2010). Here, we describe the generation and characterization of a murine mAb raised against the canine TF (cTF) ectodomain for potential future test development for TF detection in native and neoplastic canine blood, tissues and cells or further characterizing TF expression in normal and diseased canine tissue.

2. Materials and methods

Tissue factor was cloned from Madin-Darby canine kidney (MDCK) cells, as we have previously described (Stokol et al., 2011). Methods for protein expression and mAb production have been described previously in detail (Wagner et al., 2003; Wagner et al., 2005). In brief, the insert was sub-cloned into the mammalian expression vector (pcDNA3.1 (−)/Myc-His, version B, Invitrogen, Carlsbad, CA, USA) containing equine IL-4 (eIL-4) (Wagner et al., 2012). Recombinant cTF was expressed as an eIL-4 fusion protein (rcTF-eIL-4) in Chinese Hamster Ovarian (CHO) cells and purified from serum-free media using an anti-eIL-4 affinity column (Wagner et al., 2012). A BALB/C mouse was immunized as previously described in detail (Wagner et al., 2003), using 50 μg purified rcTF-eIL4 protein initially followed by 12.5 μg protein booster injections, with an adjuvant (Adjuvant MM, Gerbu, Heidelberg, Germany), for the first 3 immunizations. MAbs were generated by fusion of splenic B cells from the immunized mouse and murine myeloma cells (Wagner et al., 2003). Increased serum anti-fusion product titers were confirmed with an anti-eIL-4 ELISA. Spleen cells were fused to X63-Ag8.653 myeloma cells, then culture supernatants were tested for mAb to the rcTF-eIL4 fusion protein by ELISAs using the purified fusion protein or purified recombinant eIL-4 as Ag in parallel, as previously described (Wagner et al., 2012). Single cell clones detecting rcTF-eIL4 but not eIL-4 were transferred into individual wells of 96 well plates. Supernatants were screened using flow cytometry with 0.5% saponin-permeabilized CHO cells expressing rcTF-eIL-4 or eIL-4 and immunohistochemical staining of tubules in frozen canine kidney sections. A single mAb, #133-2 (isotyped as IgG1) was purified with a protein G column and characterized by reactivity with canine cancer cells, normal canine tissues (kidney, intestine, skin and brain) and LPS-stimulated PBMCs, using immunologic techniques as outlined below. All reagents were from Sigma-Aldrich (St Louis, MO), unless specified.

For canine cancer cell lines, we used CMT25 mammary tumor and HMPOS osteosarcoma cells that express high and low TF, based on labeling with a rabbit polyclonal anti-human TF Ab (Stokol et al., 2011). Cells were cultured in L-15 (CMT25) or RPMI-1640 (HMPOS) media, supplemented with 10 mM HEPES (CMT25), 10% FBS (20% for CMT25), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U), and streptomycin (100 μg). Cells were detached with 0.25% trypsin-EDTA and viability was >90% with trypan blue exclusion (Stokol et al., 2011). Tissue culture reagents were from Life Technologies (Grand Island, NY), except for FBS (Thermo-Scientific, Rockford, IL). PBMCs were isolated from the blood of clinically healthy dogs using double-density gradient centrifugation (Histopaque 1.070 and 1.119), as described (Ogasawara et al., 2012). Cells were plated in 48-well culture plates (1×10^6/well) in RPMI-1640 with 10% FBS, L-glutamine and penicillin-streptomycin. After 1 h, wells were washed with PBS to remove loosely adherent cells and cells were cultured in media overnight. The following day, cells were stimulated for 4 h with 1 μg/mL LPS, using PBS as a vehicle control.

For flow cytometry, detached cancer cells (5×10^5 cells/reaction) were incubated with 20 μg/mL mAb #133-2 or isotype control (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), followed by an Alexa488 (A488)-conjugated donkey anti-mouse IgG (1:200, Life Technologies), both for 15 min on ice. We have previously used a rabbit polyclonal anti-human TF Ab to describe TF expression on canine cancer cells (Stokol et al., 2011), thus we used this same polyclonal Ab (20 μg/mL, Sekisui Diagnostics, Exton, PA) as a positive control for TF expression with a rabbit IgG negative control (Jackson ImmunoResearch) followed by an A488 donkey-anti-rabbit IgG (1:200, Life Technologies). Analysis was done on 10,000 cells (FACSCalibur™, BD Biosciences, Franklin Lakes, NJ) as described (Stokol et al., 2011).

For immunohistochemical staining of canine tissues, instructions of a commercial kit were followed (ImmPRESS Peroxidase anti-mouse polymer detection kit, Vector Laboratories, Burlingame, CA). In brief, cryostat frozen sections were fixed in ice-cold acetone, washed, then endogenous peroxidase was quenched with 0.5% hydrogen peroxide. Sections were incubated with 20 μg/mL #133-2 or isotype control overnight at 4 °C, followed by 1 h at 37 °C. Ag localization was visualized with AEC substrate (Life Technologies), with light hematoxylin counterstaining.

Immunofluorescent microscopy was performed on cancer cells (1×10^5 on fibronectin-coated coverslips) and PBMCs (in 48 well plates, washed after LPS or PBS exposure) as previously described (Stokol et al., 2011), using 20 μg/mL mAb #133-2 or isotype control.

For immunoblotting, cultured cells were lysed in RIPA buffer (50 mm Tris–HCl, 150 mm sodium chloride, 1% Nonidet P40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA) containing protease inhibitors (2 μM leupeptin, 10 μM aprotonin, 1 mM phenylmethylsulfonyl fluoride). Snap-frozen canine brain was ground with a mortar and pestle in PBS with 2 mM EDTA and protease inhibitors (Halt protease inhibitor cocktail, Thermo-Scientific, Rockford, IL). Ground tissue was centrifuged at $30{,}000 \times g$ for 20 min at 4 °C and the pellet was sonicated in PBS with 1% sodium-dodecyl-sulfate. Protein concentrations were determined with a commercial kit (DC protein assay, Thermo-Scientific). Separation of proteins was performed with a 10% SDS-PAGE gel and mAb #133-2 (10–15 μg/mL) was applied overnight at 4 °C. Signal was detected with a horseradish-peroxidase-based chemiluminescent substrate (Supersignal West Pico, Thermo-Scientific) and autoradiographic or chemiluminescent (ChemiDoc™ MP system, Bio-Rad) exposure.

To test if the mAb was inhibitory for TF procoagulant activity, we incubated CMT25 cancer cells with mAb #133-2 and performed a two-stage amidolytic assay for generation of activated coagulation factor X, as described (Ogasawara et al., 2012; Stokol et al., 2011).

Experiments were repeated a minimum of 3 separate times. Animal use protocols (blood collection for PBMCs, tissue collection for immunohistochemical staining) were approved by the Institutional Animal Care and Use Committee at Cornell University.

3. Results and discussion

Based on initial screening of cultured hybridoma supernatants, using purified fusion protein and purified eIL-4 as positive and negative selection markers, flow cytometry on permeabilized CHO cells transiently transfected with cTF-eIL4 or eIL-4, and immunohistochemical staining of renal tubules in frozen canine kidney sections,