Pilot study to determine whether transient receptor potential melastatin type 8 (TRPM8) antibodies are detected in scleroderma

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ABSTRACT

Objective. *A key mediator in cold-sensation is the protein transient receptor potential melastatin 8 (TRPM8), which is expressed on sensory nerves and cutaneous blood vessels. These receptors are activated by cold temperatures and play a key role in body thermoregulation. Cold sensitivity and Raynaud's phenomenon are frequent clinical features in scleroderma, and are thought to be secondary to a local defect in cutaneous thermoregulation. We investigated whether autoantibodies targeting TRPM8 were present in the sera of patients with scleroderma as evidence for a possible mechanism for an acquired immune mediated defect in thermoregulation.*

Methods. *Sera from 50 well-characterised scleroderma patients with Raynaud's phenomenon were studied. TRPM8 autoantibodies were assayed as follows: 1. immunoprecipitation with ^{35}S-methionine-labelled TRPM8 generated by in vitro transcription and translation, 2. immunoblotting lysates made from cells transiently transfected with TRPM8 cDNA, 3. immunoprecipitation of TRPM8 transfected lysates with detection by blotting and 4. flow cytometry.*

Results. *Fifty scleroderma patients with Raynaud's phenomenon (41 female, 39 Caucasian, 23 with limited scleroderma, and 20 with history of cancer) were studied. Four different methods to assay for TRPM8 antibodies were set up, optimised and validated using commercial antibodies. All 50 scleroderma patients' sera were assayed using each of the above methods, and all were negative for TRPM8 autoantibodies.*

Conclusions *Antibodies against TRPM8 are not found in scleroderma patient sera, suggesting that the abnormal cold sensitivity and associated abnormal vascular reactivity in scleroderma patients is not the result of an immune process targeting TRPM8.*

Introduction

A striking clinical feature of systemic sclerosis (scleroderma) is that patients acquire intolerance to cold temperatures prior to or at the onset of other disease manifestations. Signs of dysfunctional vascular thermoregulation, clinically presenting as Raynaud's phenomenon (RP), occur at disease onset in almost every patient (1). While the pathogenesis of RP is not completely understood, there is evidence that cutaneous vasoconstriction and the sympathetic response to local cooling is amplified. Vascular reactivity is regulated by intrinsic vascular mediators and extrinsic factors including the neural control of vascular tone affecting thermoregulatory vessels. These regulators interact, creating a delicate balance that provides important physiological responses including regulating regional blood flow and maintaining normal core body temperature. In scleroderma, abnormal vasoreactivity and RP are caused by the disease process disrupting thermoregulatory vessels and usual neurovascular control (1).

The normal perception of temperature is a critical function of the somatosensory system that protects us from extreme environmental temperatures (2, 3). Temperature sensitive ion channels on specialised dorsal root ganglion neurons allow cutaneous nerves to respond to both heat and cold temperature (3). To date, 6 different thermo-sensitive receptors, all members of the transient receptor potential (TRP) ion channel family, have been identified (3). TRPV1-4 have partially overlapping functions over a warm to hot thermal range, whereas TRPM8 and possibly TRPA1 cover cooler temperatures (2, 3). Primary afferent neurons containing these receptors convert thermal stimuli into action potentials that relay sensory information from the skin to

the central nervous system (4). In addition, TRPM8 receptors are expressed on vascular smooth muscle, and activation may alter cutaneous blood flow by mediating vasodilatation or vasoconstriction (5). Additionally, data suggests that activation of TRPM8 on cutaneous sensory nerves after cold temperature exposure may activate thermogenesis from body fat, thus resulting in local heat production (6). An acquired defect in TRPM8 function could therefore explain an abnormal response to cold exposure in patients with scleroderma.

We hypothesised that RP in scleroderma may in part be the clinical manifestation of altered TRPM8 expression or function due to an immune-mediated process. In this pilot study, we tested whether autoantibodies to TRPM8 were detectable in sera from scleroderma patients who had definite cold-induced vasospasm typical of RP.

Methods

Banked sera from 50 patients who met 1980 American College of Rheumatology (ACR) classification criteria for scleroderma (7) and had known RP were selected for study. A subset (n=20) of scleroderma patients with a history of cancer was included as TRPM8 is upregulated in multiple malignancies (8). Severity of RP was scored using the Medsger scale of RP with <2 being RP alone and ≥2 indicating the presence of digital pits, ischaemic ulcers or loss. All subjects were enrolled in protocols approved by the Johns Hopkins IRB.

Antibody assays

The following four different assays were used to test for TRPM8 antibodies.

• *Method 1*

Immunoprecipitation using TRPM8 generated by *in vitro* transcription and translation ("IVTT IP"). cDNA encoding FLAG-tagged full-length human TRPM8 was purchased (Origene). ^{35}S-methionine-labelled TRPM8 was generated by IVTT using a kit (Promega) from this cDNA. The radiolabelled product was then used to test for TRPM8 antibodies in patient sera as described (9). Immunoprecipitates were electrophoresed on SDS-polycrylamide gels and visualised by fluorography.

Table I. Characteristics of the 50 study participants.

Variable	Value
Age (years), mean (SD)	56.4 (13.5)
Female gender, n. (%)	41 (82)
Race, n. (%)	
White	39 (78)
Black	6 (12)
Other/Unknown	5 (10)
Scleroderma subtype, n. (%)	
Limited	23 (46)
Diffuse	27 (54)
Scleroderma disease duration at time of serum sampling (years)*, mean (SD)	9.8 (11.3)
Raynaud's duration at time of serum sampling (years), mean (SD)	11.5 (11.3)
Severe Raynaud's phenomenon**, n. (%)	23 (46)
History of digital gangrene, n. (%)	3 (6)
Pulmonary hypertension^, n. (%)	13 (26)
Baseline modified Rodnan skin score, mean (SD)	11.5 (10.8)
Maximum modified Rodnan skin score, mean (SD)	13.7 (12.2)
Abnormal Medsger General Severity Score^^, n. (%)	30 (60)
History of gastrointestinal disease#, n. (%)	42 (84)
History of renal crisis, n. (%)	4 (8)
History of tendon friction rubs, n. (%)	10 (20)
History of myositis, n. (%)	10 (20)
Baseline cardiopulmonary function, mean (SD)	
Forced vital capacity (% predicted), n=44	79.5 (16.0)
Diffusing capacity (% predicted), n=42	74.5 (20.1)
Right ventricular systolic pressure (mmHg), n=29	34.1 (7.8)
Autoantibody status, number ever positive/number tested	
Anti-centromere	9/49
Anti-topoisomerase 1	8/49
Anti-RNA polymerase III	17/41
History of cancer, n. (%)	20 (40)

*defined as time since 1st non-Raynaud's scleroderma symptom; **severe Raynaud's phenomenon defined by a Medsger Raynaud's severity score ≥2; ^pulmonary hypertension defined by estimated RVSP ever ≥45 mmHg on echocardiography; ^^defined as Medsger general severity score ≥1 (reflecting anemia and weight loss); #defined as Medsger gastrointestinal severity score ≥1 (reflecting use of medications for reflux, an abnormal small bowel series, use of antibiotics for bacterial overgrowth, malabsorption, pseudo-obstruction or use of total parenteral nutrition).

• *Method 2*

Immunoblot of transfected lysates. HEK293 cells were transiently transfected with TRPM8 cDNA using Lipofectamine 2000, per the manufacturer's protocol (Invitrogen). Lysates were prepared by harvesting the cells in Buffer A (1% nonidet P-40, 20mM Tris pH 7.4, 150mM NaCl, 1mM EDTA and protease inhibitors). Gel samples were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with patient sera (1:3,000), followed by secondary antibody and detection by enhanced chemiluminescence (Thermo Scientific). Positive controls were performed using an anti-FLAG monoclonal antibody (1:5,000, Agilent Technologies) or a polyclonal anti-TRPM8 antibody (1:1,000, Novus), followed by appropriate secondary antibodies.

• *Method 3*

"IP/Blot". Lysates made from HEK 293 cells transiently transfected with TRPM8 cDNA (as per *Method 2*) were used for immunoprecipitations. 50µg amounts of transfected lysates (in 1ml) were incubated overnight at 4°C with 3µl patient serum. Positive controls were performed using 10µg transfected lysate with 1µg anti-FLAG or anti-TRPM8 antibody. After adding immobilised Protein A/G (Thermo Scientific), the immunoprecipitates were electrophoresed, transferred to membranes and immunoblotted as described above, using a monoclonal anti-FLAG antibody (1:5,000) as the primary immunoblotting antibody.

• *Method 4*:

Flow cytometry. HEK293 cells were transiently transfected with TRPM8 (C-terminal FLAG tag) or empty vector

(negative control) cDNA per Method 2. Incubations with patient sera (diluted 1:320 in PBS pH 7.4, 1% FBS) were performed at 4°C for 30 minutes, followed by phycoerythrin (PE)-conjugated anti-human IgG (1:300, Sigma) at 4°C for 15 minutes. Positive controls were performed by staining permeabilised cells (Intracellular Fixation and Permeabilisation Buffers, eBioscience) with an anti-FLAG antibody (1:1000 in permeabilisation buffer, 1% FBS) at 4°C for 15 min followed by PE-conjugated anti-mouse IgG antibody (1:500, Sigma) at 4°C for 30 min. Gates were based on negative control cell staining. Data were collected using a BD FACS-Aria Cell Sorter (BD Biosciences) and analysed using FCS Express (De Novo Software).

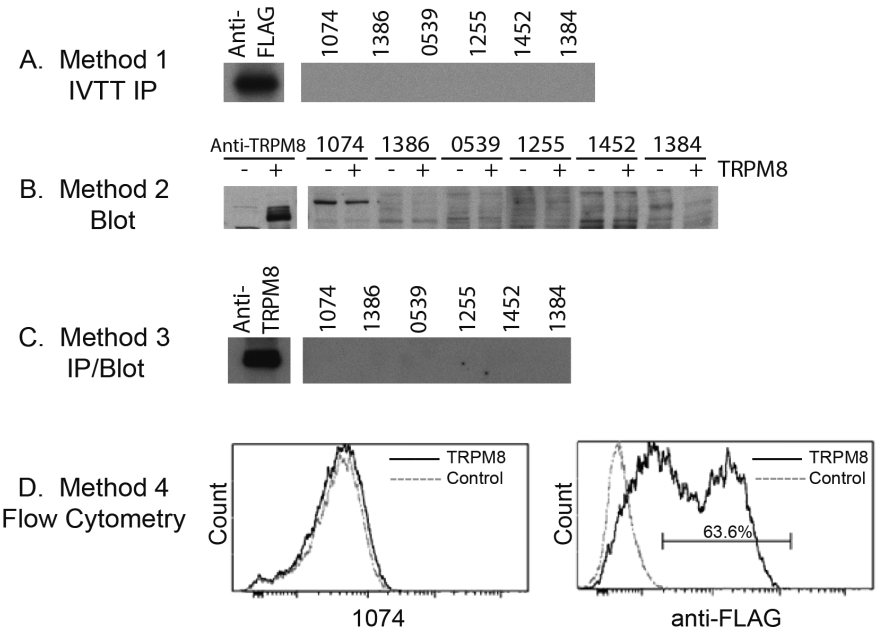

Fig. 1. Three different assays were used to test for TRPM8 antibodies in scleroderma patient sera. **A:** FLAG-tagged, ^{35}S-methionine-labelled *in vitro* transcription and translated (IVTT) TRPM8 was immunoprecipitated with anti-FLAG monoclonal antibody (left lane) or 5 different scleroderma sera. **B:** Equal protein amounts of lysates made from HEK293 cells transfected with TRPM8 cDNA ("+") or vector alone ("-") were immunoblotted with a polyclonal anti-TRPM8 antibody (left panel), or scleroderma patient sera (right panel).
C: TRPM8 transfected lysates were immunoprecipitated with polyclonal anti-TRPM8 (left panel) or scleroderma patient sera (right panel), then immunoblotted with anti-FLAG antibody to detect the precipitates.
D: HEK293 cells were transiently transfected with TRPM8 cDNA or vector alone (control) and incubated with scleroderma patient sera (left panel). Incubation with a monoclonal anti-FLAG antibody (right panel) served as a positive control.

Results

Fifty scleroderma patients were studied (Table I). Twenty-three had a history of severe RP with history of digital pits, ischaemic ulcers or loss prior to serum sampling.

We first tested for antibodies against TRPM8 using IVTT IP, with ^{35}S-methionine labelled TRPM8 as source material (*Method 1*). This is an assay we have used successfully many times to assay for various antibody specificities (9-12). Although both anti-FLAG and anti-TRPM8 antibodies immunoprecipitated the IVTT TRPM8 well, none of the scleroderma sera did so (Fig. 1A and not shown).

Since TRPM8 is a transmembrane protein, it was possible that the IVTT product was incorrectly folded. We therefore designed alternate assays to test for these antibodies that used endogenously synthesised TRPM8 as the source material *(Methods 2-4)*.HEK293 cells were transiently transfected with cDNA encoding FLAG-tagged human TRPM8. Gel samples were prepared in several different ways: in the absence or presence of reducing agent, and were either boiled (3 mins) or kept at room temperature (20 mins) before loading on gels. Using all of these conditions, immunoblotting with anti-TRPM8 or anti-FLAG antibodies (Fig. 1B and not shown) confirmed robust TRPM8 expression in these lysates. In contrast, none of the scleroderma sera immunoblotted TRPM8 in these lysates, irrespective of how the gel samples were prepared (*Method 2*, Fig. 1B).

Since most antibodies immunoprecipitate, but not all immunoblot, the third approach we used was based on immunoprecipitation of endogenously synthesised TRPM8. The TRPM8 transfected lysates (validated above) were used for immunoprecipitations, followed by detection with immunoblotting using anti-FLAG antibody. Although this approach worked well using the anti-TRPM8 antibody to immunoprecipitate, none of the scleroderma sera immunoprecipitated TRPM8 from these lysates (Fig. 1C). We also radiolabelled TRPM8 transfected cultures and immunoprecipitated using the commercial anti-TRPM8 antibody as well as the scleroderma sera, with visualisation by fluorography. These results were identical to those obtained above (IP followed by blotting for detection) – only the commercial antibody immunoprecipitated TRPM8.

To maintain the structural integrity of TRPM8 within the cell membrane, flow cytometry was used as a fourth technique to detect anti-TRPM8 antibodies. Staining with anti-FLAG antibody confirmed robust TRPM8 expression in transfected cells, but antibodies against TRPM8 were not detected in any of the patient sera (Fig. 1D).

Discussion

We examined whether patients with scleroderma, an autoimmune disease with prominent vascular manifestations, had autoantibodies to the cold receptor TRPM8. Autoantibodies to TRPM8 were not detectable in scleroderma patients' sera, including those with severe RP. This pilot study, using several state-of-the-art methods to detect autoantibodies, failed to provide evidence that these receptors are a primary autoimmune target in scleroderma. Since TRPM8 is upregulated in multiple malignancies (8), a subset of scleroderma patients with a history of

cancer was included in this study; autoantibodies against TRPM8 were not detected in these patients.

Interestingly, expression of TRP channels is well described in sensory nerve cells and vascular smooth muscle, and in other cell types and organs, including respiratory and gastrointestinal tract (13, 14), that are targeted in scleroderma. Activation of TRPM8 in lung cells by exposure to cold or menthol is reported to enhance expression of a range of pro-inflammatory cytokines (15). In animal models, TRPM8 is detected in the gastrointestinal tract and is thought to play a role in motility, absorptive and secretory processes (14). Therefore, it was thought possible that an acquired immune mediated defect in TRPM8 function might explain in part the abnormal thermoregulation of RP and malfunction in other organs such as the lung and gastrointestinal tract. The absence of autoantibodies against the TRPM8 does not support this view.

Possible explanations for the absence of TRPM8 autoantibodies include long disease duration (16, 17) and immunosuppressive therapies used to treat scleroderma and/or cancer. Since 40% of the population we studied had a short scleroderma disease duration (<2 years), 44% of patients did not have antecedent immunosuppressive drug exposure prior to the serum sample draw, and only 12% of patients had been exposed to chemotherapies, we believe it is unlikely that these factors account for the lack of TRPM8 autoantibodies.

This pilot study is a cross-sectional survey of selected scleroderma patients that focused on an immune response to one member of a family of TRP receptors. While we did not demonstrate that the immune system is targeting TRMP8, it is possible that other members of the TRP ion channel family are targeted or that non-immune perturbation of these channels can disturb normal physiological responses to ambient temperatures. Further studies will be needed to address this important mechanistic question.

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